TALITA DINIZ MELO HANCHUK

CARACTERIZAÇÃO FUNCIONAL: A CINASE HUMANA NEK5 INTERFERE NEGATIVAMENTE NA MORTE CELULAR E NO PROCESSO DE POLIGLUTAMILAÇÃO

Functional Characterization: The human kinase Nek5 interferes negatively in cell death and the polyglutamylation process

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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requerimentos exigidos para a obtenção do Título de Doutora em Biologia Funcional e Molecular, na área de Bioquímica.

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Orientador/ Supervisor: Prof. Dr. Jörg Kobarg

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Resumo

Membros da família das Neks são cruciais para o início da mitose em eucariotos. Têm sido funcionalmente atribuídas a todas as 11 Neks humanas uma das três principais funções estabelecidas para esta família em mamíferos: (1) centríolos / divisão celular; (2) funções no cílio primário / ciliopatias; e (3) resposta à danos no DNA (DDR). No artigo de revisão (artigo I), relatamos uma análise detalhada atual sobre cada uma das 11 Neks. A hipótese é que as Neks possam conectar elementos reguladores que permitem o refinamento e a sincronização de eventos celulares. Dentre os membros desta família, Nek5 é a cinase mais negligenciada. Ensaios de duplo híbrido em leveduras (Y2H) foram realizados para identificar e caracterizar parceiros de interação Nek5; e proteínas mitocondriais foram observadas (artigo II). Ensaios de apoptose mostraram efeitos protetores na morte celular após tratamento com tapsigargina (2 μM) de células HEK293T que superexpressam a hNek5, bem como a diminuição na formação de Espécies Reativas de Oxigênio após 4 horas de tratamento. A atividade da cadeia respiratória mitocondrial estava diminuída após superexpressão de hNek5, especialmente nas etapas de transferência de elétrons do TMPD para o citocromo c e no complexo II. O Y2H permitiu também a identificação da poliglutamilase de proteínas TTLL4 como um parceiro de Nek5 (artigo III). Células silenciadas para a Nek5, assim como células que expressam a versão "*kinase dead*" de Nek5, apresentaram por western blot e ensaio *in vitro* de atividade poliglutamilação um aumento na poliglutamilação de proteínas após transfecção com TTLL4. Em conclusão, nossos dados sugerem pela primeira vez a localização mitocondrial e a participação de Nek5 na morte celular e no processo poliglutamilação diminuindo a atividade de TTLL4 através de sua fosforilação inibitória.

Abstract

Members of the Nek Family are crucial for the initiation of mitosis eukaryotes. All 11 human Neks have been functionally assigned to one of the three core functions established for this family in mammals: (1) centrioles/mitosis; (2) primary ciliary function/ciliopathies; and (3) DNA damage response (DDR). In the core section of the review (article I), we report the current detailed functional knowledge on each of the 11 Neks. We raise the hypothesis that Neks may be the connecting regulatory elements that allow the cell to fine tune and synchronize cellular events. Nek5 is the most neglected among members of the Nek kinases family. A yeast two-hybrid (Y2H) screen was performed to identify and characterize Nek5 interaction partners and mitochondrial proteins were retrieved (article 2). Apoptosis assay showed protective effects of hNek5 over-expression from Hek293-T's cell death after thapsigargin treatment (2 μM) as well as an increase in ROS formation after 4 hours of treatment. Mitochondrial respiratory chain activity was found decreased upon hNek5 over-expression especially at the electrons transfer steps from TMPD to cytochrome c and at the complex II. The yeast two-hybrid allowed also the identification o TTLL4 as a Nek5 partner (article 3). Nek5 silenced cells as well as cells expressing a "kinase dead" version of Nek5, displayed an increase in polyglutamylation of proteins after TTLL4 transfection by western blot and *in vitro* polyglutamylation activity assay. In conclusion, our data suggest for the first time mitochondrial localization and functions for Nek5 and its participation in cell death and cell respiration regulation. This work also showed the function of Nek5 in the polyglutamylation process decreasing the role of TTLL4 through inhibitory phosphorylation by Nek5.

Sumário

A cada dia um desafio; em alguns desafios, alguns erros; a cada erro a oportunidade de aprender e de não errar novamente.

Quezia.

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Lista de Abreviações

Aa: aminoácidos

ATM: *Ataxia telangiectasia mutated*

ATR: *Rad3-related protein*

BCLAF-1: *Bcl-2-associated transcription factor 1*

BSA: Albumina bovina sérica (*Bovine serum albumine*)

cDNA: DNA complementar

Cdk: Kinase dependente de ciclina (*Cyclin-dependent kinase)*

C-Nap1: *Centrossomal Nek2-associated protein 1*

Cox11: *Cytochrome c oxidase assembly protein COX11, mitochondrial*

DDR: resposta de dano de DNA (*DNA Damage Response*)

DNA: Ácido Desoxirribonucleico

DTT: Dithiothreitol

EDTA: Ácido etilenodiaminotetraacético

FACT: *facilitates chromatin transcription*

HeLa: *Human epithelial cervical cancer cell line*

HEK293: *Human Embrionyc Kidney 293 Cell line*

HEK93T: HEK293 que expressa o antígeno T do SV40 constitutivamente.

IAA: Iodacetamida

IP: Imunoprecipitação

kDa: kilo Dalton

MCF-7: *Human breast adenocarcinoma cell line*

mRNA: RNA mensageiro

MS: Espectrometria de massas (*mass spectrometry*)

MT: Microtúbulos

MTCO: Centro organizador de microtúbulos (*Microtubule-organizing center*)

MTX2: *Metaxin-2*

MW: *Molecular weight*

Nek: *NIMA Related Kinase*

NIMA: *Never in mitoses gene* A

NLS: *Nuclear localization signal*

PARP: *Poly [ADP-ribose] polymerase* 1)

PCR: Reação em cadeia da polimerase (*Polymerase chain reaction*)

PEI: *Polyethyleneimine*

PI: Iodeto de propídeo

p53: Proteína tumoral 53kDa

PK: proteinas cinases

PKC: Proteína Kinase C

PKD: Doença policística renal (*Polycistic Kidney Disease*)

Plk: *Polo-like kinases*

PMSF: Fluoreto de fenil metil sulfonamida

PEST: Prolina, glutamato, serina e treonina

PVDF: Polivinildifluoridina

RCC1: Regulador de condensação cromossômica (*Regulator of Chromosome Condensation*)

RE: Retículo Endoplasmático

ROS: Espécies reativas de oxigênio (*Reactive oxigen species*)

SAC: spindle assembly checkpoint

SDS-PAGE: *Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis*

shRNA:*Small hairpin* RNAs

TTLL: *Tubulin Tyrosine Ligase-Like*

TTLL4: *Tubulin polyglutamylase TTLL4*

U2-OS*: Human Bone Osteosarcoma Epithelial Cells*

Introdução

As Proteínas cinases e a Proteína NIMA de *Aspergillus nidulans*

Proteína-cinases (PKs) estão entre as maiores famílias de genes em eucariotos e representam entre 1,5 a 2,5 % dos genes (Manning *et al.*, 2002). Elas medeiam a maioria dos eventos de transdução de sinal em células eucarióticas, seja pela alteração da atividade ou localização do substrato, interação com outras proteínas ou pelo controle de inúmeros processos celulares incluindo metabolismo, transcrição, progressão do ciclo celular, rearranjo do citoesqueleto, movimento celular, apoptose e diferenciação (Manning *et al.*, 2002). PKs que atuam na regulação do ciclo celular, principalmente nos *checkpoints*, têm sido consideradas promissoras como alvos terapêuticos, principalmente para o câncer. Os principais *checkpoints* foram identificados no final da fase G1 (ponto de restrição), na passagem de G2 para mitose e dentro da própria mitose (na metáfase). As PKs que atuam nestes *checkpoints* incluem as CDKs (*cyclin dependent kinases*), cinase Aurora, cinases tipo- Polo, ATR/ATM e as NEKs (para revisão Malumbres e Barbacid, 2007; Lapenna e Giordano, 2009). Perdas ou falhas nestes pontos de controle vêm sendo associadas com o desenvolvimento de células neoplásicas, uma vez que essas não obedecem mais o ritmo de divisão celular e apresentam um acúmulo de erros no DNA (para revisão Malumbres e Barbacid, 2007).

Entre as diversas famílias de PKs descritas, os membros da família relacionados à NIMA (*never* i*n* *mitosis, gene A*) são os menos estudados e caracterizados funcionalmente. A proteína NIMA, foi identificada por Ron Morris (1975), no fungo *Aspergillus nidulans*, em um *screening* genético de mutantes sensíveis a temperatura que falharam na progressão do ciclo celular. Foram identificados mutantes que bloquearam as células em mitose (*bim: blocked in mitosis*) ou na intérfase, nunca entrando em mitose (*nim: never in mitosis*). NimA foi a primeira proteína identificada e consiste de uma proteína serina/treonina cinase crítica para a progressão do ciclo celular, sendo necessária no início da mitose (Bergen *et al*., 1984). Mutantes de NIMA sensíveis à temperatura ou a superexpressão de formas dominante-negativas de NIMA causam interrupção do ciclo celular na fase G2, detectando-se DNA não-condensado e microtúbulos na forma encontrada em intérfase (Osmani *et al*., 1991). Além disso, a superexpressão de NIMA em fungos e em células de mamíferos resultou no desencadeamento precoce de eventos relacionados à mitose, incluindo a condensação da cromatina e a despolimerização dos

microtúbulos (Lu & Hunter, 1995). De Souza et al demonstraram em 2000 que a condensação de cromatina observada após a superexpressão de NimA ocorre via fosforilação da histona H3. A habilidade de NIMA em regular a mitose em eucariotos superiores sugere a existência de vias de sinalização envolvendo proteínas homólogas à NIMA, conservadas durante a evolução.

NimA related kinases - Neks

Os grupos de Pawson e Nigg foram os primeiros a identificar em mamíferos, proteínas similares a NIMA, denominaram-nas Nek (*NIMA related kinases*) (Letwin et al., 1992; Schultz & Nigg, 1993). Através de sequenciamento genômico foram identificados 11 genes que codificam para diferentes proteínas da família das Neks (Manning et al., 2002; Forrest et al., 2003). As Neks formam uma família altamente conservada de proteínas cinases envolvidas na regulação do ciclo celular. As Neks de mamíferos podem estar localizadas nos cílios, centrossomos, núcleo, citoplasma ou mitocôndrias e podem, dentre outras funções, afetar a progressão do ciclo, além de funções ciliares (Quarmby & Mahjoub, 2005). Em mamíferos, as proteínas cinases estruturalmente homólogas à NIMA apresentam em torno de 40 - 45% de homologia com NIMA em seu domínio catalítico. A extremidade carboxi-terminal (C-terminal) constitui o domínio regulatório e apresenta maior variabilidade, estando envolvida na determinação da especificidade por substratos e na interação com outras proteínas (Surpili et al., 2003; O'Connel et al., 2003; O'Regan et al., 2007; Moniz et al., 2011; Fry et al., 2012). Esta diversidade na região C-terminal pode ser fundamental para as funções distintas de cada membro da família.

Dentre as proteínas da família das Neks, Nek2 é até o momento a melhor caracterizada. As proteínas Nek2 apresentam maior homologia a NIMA, aproximadamente 47% (Fry, 1999), embora células *knockdown* para Nek2 consigam entrar em mitose (Fletcher et al., 2005). A estrutura dessa proteína apresenta um domínio *coiled coil* logo após o domínio catalítico e esse domínio é um sítio de dimerização que facilita a autofosforilação (Fry, 1999). Ainda está presente um sítio para ligação da proteína PP1, sendo que uma vez ocupado ocorre defosforilação da Nek2 e sua conseqüente inibição (Rellos et al., 2007). Em vertebrados Nek2 regula a separação dos

centrossomos durante a fase G2/M do ciclo celular, sendo de fundamental importância para a formação do fuso mitótico e adequada separação dos cromossomos (Quarmby & Mahjoub, 2005; Fry et al., 1999; Hames & Fry, 2002). Nek2 também pode desempenhar um papel adicional nos pontos de controle (*checkpoints*), que são momentos do ciclo celular nos quais uma maquinaria de proteínas confere se o ciclo está se desenvolvendo corretamente e a partir dessa verificação a célula recebe sinais de que deve continuar o processo ou que deve parar e ativar a cascata sinalizadora de apoptose. Defeitos nos componentes dessas maquinarias podem acarretar no desenvolvimento de doenças, como cânceresdevio ao acúmulo de erros e mutações.

Nek8 e Nek1 são produtos de genes relativos a patologias, como a doença policística do rim (PKD, Polycystic Kidney Disease). Suas funções celulares envolvem, possivelmente, uma via de sinalização relacionada aos cílios e ao ciclo celular (Quarmby & Mahjoub, 2005). Nek8 é superexpressa em tumores mamários humanos (Bowers & Boylan, 2004), assim como Nek3 (Miller et al., 2005). A Nek3 humana parece apresentar um importante papel na sinalização do receptor de prolactina, especificamente em uma via que contribui para a progressão e motilidade do câncer de mama (Miller et al., 2005). Adicionalmente, Chen e colaboradores (1999) indicam a participação da proteína Nek4 murina na divisão celular meiótica. Aliado a esses estudos, Reininger e colaboradores (2005) demonstraram que Nek4 é necessária para a conclusão do ciclo sexual do *Plasmodium*, parasita da malária.

As proteínas Nek6 e Nek7 são os membros menores e estruturalmente mais simples entre as Neks conhecidas. São constituídas quase que somente por seus domínios catalíticos que se localizam na região C-terminal e, ao contrário das demais Neks, apresentam uma curta extremidade N-terminal (O'Connel *et al*., 2003). Nek6 e Nek7 compartilham 87% de identidade nos seus domínios catalíticos e, juntamente com a proteína F196H6.1 de *Caenorhabditis elegans*, têm sido descritas como representantes de uma subfamília altamente conservada de Neks (Kandli *et al*., 2000). A Nek6 endógena é ativada durante a mitose, concomitante com um aumento do seu nível de expressão (Belham *et al*., 2003), enquanto a Nek7, ao contrário, parece ser relativamente constante ao longo do ciclo celular (Kim *et al*., 2007). Além disso, foi descrito uma localização de Nek7 no centrossomo de células U2OS e HeLa tanto na intérfase quanto na mitose

(Yassachar *et al*., 2006; Kim *et al*., 2007), enquanto a Nek6 apresenta uma distribuição difusa, principalmente citoplasmática. Contudo, a superexpressão de mutantes cataliticamente inativos das Neks 6 e 7 produzem fenótipos similares, como células exibindo elevados índices mitóticos, defeitos no fuso mitótico, anormalidades nucleares e apoptose (Yin *et al*., 2003; Yassachar *et al*., 2006). Esses fenótipos são também observados a partir da depleção das Neks 6 e 7 em células HeLa, utilizando-se iRNA, ocorrendo a retenção das células na metáfase, condensação normal da cromatina e alinhamento dos cromossomos, mas incapacidade de completar a segregação dos mesmos. A atividade das Neks 6 e 7, portanto, parece ser necessária para a progressão da anáfase, onde as células são retidas no ponto de controle do fuso mitótico e entram em apoptose, ou completam a mitose, mas com a aquisição de anormalidades nucleares durante o processo (Yin *et al*., 2003; Yassachar *et al*., 2006; Kim *et al*., 2007). Além da semelhança estrutural, ao que tudo indica, as NEKs 6 e 7 participam de uma mesma cascata de sinalização na mitose, onde NEK6 parece ser ativada por outra NEK, a NEK9 (Belham *et al.*, 2003; Roig *et al.*, 2002).

A Nek9, por sua vez, é uma das maiores Neks e seu domínio catalítico é bastante conservado. O domínio regulatório C-terminal contem uma região homóloga à RCC1, um segmento rico em Ser/Thr/Pro, e um motivo *coiled-coil* de dimerização. A região similar a RCC1 seria um possível sítio de auto-inibição da Nek9. Roig e colaboradores (2002), realizaram microinjeção de anticorpos contra Nek9, também conhecida como Nercc1, em células de cultura, o que resultou em anormalidades no fuso mitótico e desalinhamento cromossomal. Sua expressão não é alterada durante o ciclo celular, mas a atividade aumenta na mitose.

Além de Nek 1 e 8 que estão relacionadas à doença cística dos rins (Upadhya *et al.*, 2000 e Liu *et al.*, 2002), várias outras proteínas da família das Neks são produtos de genes relacionados a patologias. A expressão de muitos membros desta família estão associoadas ao câncer, Nek8 e Nek3 estão superexpressas em tumores mamários em humanos (Bowers & Boylan, 2004; Miller *et al*., 2005), e a superexpressão de Nek6 é identificada em carcinomas hepatocelulares (Chen *et al.*, 2006). O gene da Nek6 também está freqüentemente relacionado a câncer de mama, coloretal, pulmão e laringe, além de ser apontada como um marcador potencial de câncer gástrico *(Capra et al.*, 2006).

Estudos indicam que Nek6 e Nek 1 estão relacionadas aos *checkpoints* de dano de DNA durante a intérfase. Nek6 é fosforilada em resposta a danos no DNA por exposição a raios UV, fazendo que as células fiquem presas na transição G2/M da intérfase, além de ter sua atividade totalmente abolida durante a mitose (Lee et al., 2008). Do mesmo modo, células deficientes em Nek1 são defeituosas nos *checkpoints* da transição G1/S e da fase M, não reparam DNA e apresentam quebra de cromatina e cromossomos defeituosos (*Chen at al.*, 2008). Em geral, células que apresentam mutações nos genes de Nek1 e Nek6 falham ao reconhecer e reparar danos no DNA. Após indução de dano, há um aumento na expressão da NEK1 e uma migração desta do citoplasma para o núcleo, em regiões onde os danos ocorreram (Letwin *et al.*, 1992; Polci *et al.*, 2004). Ainda, células que não expressam NEK1 apresentam hipersensibilidade a efeitos letais da radiação ionizante. O mais recente membro descoberto dessa família, Nek11, foi implicado nas respostas a estresses genotóxicos e de replicação do DNA (Noguchi *et al*., 2002). A NEK7 é super-expressa em câncer de laringe e, juntamente com a NEK6, em câncer de mama e colo-retal (Capra *et al.*, 2006).

Além dessas super-expressões dos genes que codificam as NEKs humanas também já foi descoberto que eles são alvos de mutações em câncer (Greenman *et al.*, 2007). Isso sugere que mutações nelas podem conferir vantagens seletivas para sobrevivência e crescimento das células cancerígenas. Sendo assim, as NEKs não somente são objetos interessantes de estudos funcionais e estruturais, mas também ótimos candidatos para varreduras de identificação de novos inibidores que possam eventualmente ser utilizados em novas estratégias terapêuticas anti-câncer.

A tabela 1 resume as principais funções atribuídas a Neks, assim como sua localização celular e tecidos em que elas já foram identificadas como expressão alterada (doenças relacionadas).

Tabela 1: Funções propostas para Neks de mamíferos. As funções biológicas, localização celular e tecidos expressos / doenças associadas dos membros da família das Neks. DDR: Resposta de Dano de DNA. MTOC: Centro organizador de microtúbulo. PKD: Síndrome do rim policístico.

Nek	Função Biológica	Localização celular	Tecidos expressos / Doenças associadas
	Ciliogênese [1-5]	Núcleo [7]	PKD1 e infertilidade em camundongos [5]
	DDR [6-10]	Mitocôndria [14]	Alta expressão em bexiga, estômago, intestino, colo, reto, rim e testículo [15]
	Sinalização de apoptose [11]	Citoplasma [7]	Polidactilia [3]
	Regulação do ciclo celular [12]		
Nek1	Regula estabilidade de centrossomos [13]		
	Separação dos centrossomos [34, 16-20]	Centrossomos [34]	Aumento da expressão em duto biliar [36]
	Ponto de checagem em mitose [21]	MTOC [35]	Aumento da expressão em câncer de mama [37, 38] e câncer ovariano [39]
	Sinalização de SAC e alinhamento e segregação dos cromossomos [22]	Citoplasma [35]	Aumento da expressão em mieloma [40]
	Transição G2/M do ciclo celular mitótico [23- 25]	Núcleo [34]	Retinite pigmentosa [38]
	Desenvolvimento embrionário [26, 27]		Expressão relacionada a neuroblastoma [41]
	Segregação nuclear meiótica [29, 30]		Aumento da expressão em tumores de Ewing, linfomas de células B, colangiocarcinomas, adenocarcinomas de pulmão e seminomas testiculares [41 -46]
	Segregação das cromátides irmãs em mitose [31-32]		Neurofibroma plexiforme [47]
	Regulamentação de microtúbulos do fuso para os cinetócoros [22, 33]		Câncer de fígado [119], cérvix [120] e ovário [39]
Nek2			Tumor maligno da bainha do nervo periférico [47]
	Acetilação de tubulinas [48]	Citoplasma [56]	Câncer de mama [15, 57]
	Morfologia e polaridade dos neurônios [48]		Expressão reduzida em câncer de pulmão [15]
	Endocitose[49]		Alta expressão em testículo, ovário e cérebro [58]
	Sinalização da Prolactina [50, 51]		
	Divisão celular [52]		
	Organização de citoesqueleto [53]		
	Regulação de microtúbulos neuronais [54]		
Nek3	Tráfego de membranas [55]		
	Estabilização dos microtúbulos [59]	Núcleo [15]	Redução em câncer de mama, pâncreas, próstata e estômago [15]
	Formação do cílio primário [60]		Desordem bipolar [62]
	DDR [61]		Alta expressão em testículo [63]
Nek4	Regulação de senescência replicativa e resposta a dano de DNA [61]		

Nek5	Diferenciação de células musculares[64]	Não definida	Reduzido em cabeça e pescoço [15]
	Participa junto com as Neks 7 e 9 da mesma cascata de sinalização na mitose [65, 66, 67, 68]	Citoplasma [81]	Aumento da expressão em câncer gástrico [15, 83]
	A super-expressão de Nek6 correlaciona-se à super-expressão de Pin1 em 70% dos carcinomas de células hepáticas [70]	Núcleo [81]	Linfoma não Hodgkin, mama, colo retal e pulmão [15, 84]
	DDR [71]	Centrossomo [82]	Aumento em carcinoma hepatocelular [70, 113]
	Processo apoptótico [60, 72]		Aumento da expressão em esofagite erosiva e adenocarcinoma de esôfago [114]
	Segregação cromossômica [74]		Redução em câncer de próstata, renal e endometrial [15]
	Citocinese [68, 72]		
	Desmontagem do envelope nuclear [75]		
	Regulação da senescência celular [76-78]		
	Regulação do ciclo celular mitótico [79, 80]		
	Transição metáfase/anáfase [72]		
Nek6	Formação do fuso mitótico [68, 72]		
	Duplicação do centríolo [80]	Centrossomo [117]	Alta expressão em câncer de laringe, mama, colorretal e vesícula biliar [84, 85]
	Participa junto com as Neks 7 e 9 da mesma cascata de sinalização na mitose [65, 66, 67, 73]	Midbody [117]	Redução em cabeça e pescoço, próstata e estomago [15]
	Maturação do centrossomo [73]	Pontes citocinéticas [117]	Retardo no desenvolvimento [86]
	Formação do fuso mitótico [72, 66]		Catarata congênita [87]
	Separação dos centrossomos [65, 66, 115 -117]		
	Regulação da dinâmica de microtúbulos [118]		
Nek7	Citocinese [72]		
	Morfogênese de órgãos [88]	Cílio primário [94]	PKD/ câncer de mama [96]
	Função ciliar/ sinalização [89-91]	Centrossomo [91]	Mutações do gene em câncer de pâncreas [97]
	Regulação de citoesqueleto [92]	Citoesqueleto [15]	Displasia infantil [98]
	DDR [69, 68, 93]	Citoplasma [95]	Redução em renal e de estômago [15]
			Nefronoftise [99]
Nek8			Câncer de mama [96]
	Fosforila Nek6 e 7 [65, 66, 67, 73]	Fuso mitótico [91]	Associação com câncer cabeça e pescoço [106]
	Separação dos centrossomos [65, 100]	Midbody [91]	Alta expressão em LMC [106]
	Formação do fuso mitótico [73, 100]	Citoplasma [105]	
	Maturação do centrossomo [73, 100]	Núcleo [105]	
	Alinhamento e segregação da cromatina (FACT) [101]		
Nek9	Progressão do ciclo celular [100]		

	Indução da mitose catastrófica [89]		
	Cromossomos/Citocinese [65, 66, 67, 73, 102, 103]		
	Regula a progressão de G1 e S através da interação com o complexo facilitador da transcrição [104]		
Nek10	Manutenção do *checkpoint* G2/M [121]	Não definida	Câncer de mama [121]
	DDR [121]		Redução em colo retal e de estômago [15]
Nek11	Fosforilação H1, H2 e H3 e CDC25A [108]	Núcleo [36]	Redução em câncer de testículo e cerebelo [15]
	DDR/ Ponto de checagem G2/M [107 - 112]	Microtúbulos [36]	

1. Upadhya et al., 2000; 2. White e Quarmby, 2008; 3. Thiel et al., 2011; 4; Yim et al., 2011; 5. Holloway et al., 2011; 6. Surpili et al., 2003; 7. Polci et al., 2004; 8. Liu et al., 2013; 9. Chen et al., 2011; 10. Pelegrini et al., 2010; 11 Chen et al., 2010; 12. Fry et al., 1995; 13. White & Quarmby, 2008; 14. Chen et al., 2009; 15. Berglund et al., 2008; 16. Fry et al., 1999; 17. Fry et al., 2002; 18. Rapley et al., 2005; 19. Mardin et al., 2010; 20. Matsuo et al., 2010; 21. Liu et al., 2010; 22. Wei et al., 2011; 23 Jeong et al., 2013; 24. Mori et al., 2012; 25. Westwood et al., 2009; 26. Sonn et al., 2004; 27. Fry et al., 2000; 28. Spalluto et al., 2012; 29. Rhee et al., 2002; 30. Uto et al., 1999; 31. Yao et al., 2004; 32. Lou et al., 2004; 33. Jeffery et al., 2010; 34. Smith et al., 2012; 35. Wu et al., 2007; 36. Kokuryo et al., 2007; 37. Tsunoda et al., 2009; 38. Marina e Saavedra, 2014; 39. Liu et al., 2014; 40. Zhou et al., 2013; 41. Kohler et al., 2010; 42. Wai et al . 2002; 43. de Vos et al., 2003; 44. Landi et al.,2008; 45. Barbagallo et al., 2009; 46. Adréasson et al., 2009; 47. Stricker et al., 2013; 48. Chang et al., 2009; 49. Benjamin et al., 2011; 50. Miller et al., 2005; 51. Miller et al., 2007; 52. Tanaka & Nigg, 1999; 53. Miller et al., 2007; 54. Chang et al., 2011; 55. Benjamin et al., 2011; 56. Chang et al., 2009; 57. McHale et al., 2008; 58. Kimura & Okano, 2001; 59. Doles e Hemann, 2010; 60. Coene et al., 2011; 61. Nguyen et al., 2012; 62. Sklar et al., 2011; 63. Chen et al., 1999; 64. Shimizu e Sawasaki, 2013; 65. Belham et al., 2003; 66. Bertran et al., 2011; 67. Sdelci et al., 2011; 68. Fry et al., 2012; 69. Choi et al., 2013 70. Chen et al., 2006; 71. Lee et al., 2008; 72. O'Regan et al., 2009; 73. Sdelci et al., 2012; 74. Roig et al., 2002; 75. Quarmby et al., 2005; 76. Jee et al., 2013; 77. Jee et al., 2011; 78. Zhang et al., 2014; 79. Kandli et al., 2000; 80. Kim et al., 2012; 81. Hashimoto et al., 2002; 82. Meirelles et al., 2010; 83. Takeno et al., 2008; 84. Capra et al., 2006; 85. Wang et al., 2013; 86. Salem et al., 2010; 87. Lachke et al., 2012; 88. Fukui et al., 2012; 89. Mahjoub et al., 2005; 90. Smith et al.,2006; 91. Otto et al., 2008; 92. Holland et al., 2002; 93. Jackson, 2013; 94. Fukui et al., 2012; 95. Sohara et al., 2008; 96. Bowers et al., 2004; 97. Carter et al., 2010; 98. Frank et al., 2013; 99. Habbig, et al., 2012; 100. Yang et al., 2012; 101. Tan e Lee, 2004; 102. Roig et al , 2005; 103. Kaneta et al., 2013; 104. Paladino et al., 2014; 105. Pelka et al., 2007; 106. Wu et al., 2011; 107. Ahmed et al., 2009; 108. Piao et al., 2011; 109. Noguchi et al., 2002; 110. Noguchi et al.,2004; 111. Melixetian et al., 2009; 112. Sorensen et al., 2010; 113. Cao et al., 2012; 114. Kasap et al., 2012; 115. Kim et al., 2011; 116. Yissachar et al., 2006; 117. Kim et al., 2007; 118. Cohen et al., 2013; 119. Drozdov et al., 2012; 120. Koch & Wiese, 2013; 121. Moniz e Stambolic, 2011.

Nek5

Em humanos, por meio de análises de seqüenciamento genômico, o gene codificador da proteína Nek5 foi identificado no cromossomo 13 (Dunham et al. 2004). Este cromossomo carrega genes envolvidos no câncer, incluindo câncer de mama tipo 2 (BRCA2) e retinoblastoma (RB1), e é frequentemente reorganizado em células B leucêmicas (Dunham et al. 2004). Além de humanos a sequência genômica codificadora de Nek5 foi identificada em organismos como *Mus musculus, Bos taurus, Galluls gallus,*

Pan troglodytes, Ailuropoda melanoleuca, Macaca mulatta e *Pongo abelii.*

O primeiro trabalho relacionado a Nek5 foi publicado por Shimizu e Sawazaki (2013). Eles demonstraram que Nek5 é substrato para clivagem por caspase-3 na região regulatória C-terminal, promovendo diferenciação muscular e com potenciais efeitos pró-apoptóticos. Além disso, foi demonstrado que a expressão do mRNA de Nek5 aumenta durante a diferenciação muscular e o silenciamento desta proteína prejudicou o processo de diferenciação (Shimizu & Sawazaki, 2013).

Morte celular

Kerr, Wyllie e Currie (1972) postularam pela primeiras vez dois mecanismos distintos de morte celular, necrose e apoptose. De acordo com estes autores, necrose é uma forma violenta e rápida de degradação celular estimulada pelo ambiente. Ela afeta um grande número de células em uma população e é caracterizada pelo aumento no volume citoplasmático, destruição de organelas, rompimento da membrana citoplasmática e liberação de fluídos para o meio extracelular. O termo apoptose, de origem grega, é uma alusão a queda das pétalas da flores ou folhas das árvores que se assemelham a fragmentação da membrana plasmática durante o processo apoptótico. A apoptose ocorre em células individualizadas, geralmente rodeadas por células saudáveis. Após o estímulo pró-apoptótico, ocorre compactação e segregação da cromatina, condensação do citoplasma, fragmentação nuclear, convolução da membrana celular seguida da fragmentação e formação de “corpos apoptóticos”, sem liberação do conteúdo citoplasmático para o meio extracelular (Riedl & Shi, 2004; Horvitz, 1999).

A apoptose é importante não somente para a eliminação de células “doentes” mas também durante a organogênese, desenvolvimento embrionário, manutenção da homeostase tecidual e no sistema imune. Alterações em mecanismos de apoptose podem levar ao surgimento de doenças neurodegenerativas, autoimunes e do câncer (Wong, 2011; Portt et al., 2011). Células que sofrem mutações em genes reguladores da apoptose por exemplo, apresentam potencial oncogênico elevado, de fato, a maioria dos tumores apresentam resistência a apoptose. Diversos estímulos podem levar a sinalização para a apoptose, como por exemplo, danos ao DNA, perturbações no citoesqueleto e tratamento com drogas.

A maior parte dos processos apoptóticos têm como moléculas efetoras as proteínas caspases (proteases cisteíno-aspárticas) que são clivadas e ativadas de forma coordenada. As caspases podem ser classificadas como iniciadoras (caspases 2, 8, 9 e 10) ou efetoras (Caspases 3, 6 e 7) (Riedl & Shi, 2004; Horvitz, 1999).

Atualmente duas vias principais de apoptose foram identificadas: as vias intrínseca e extrínseca. Na via extrínseca, ou via de receptores de morte celular, destacam-se as moléculas sinalizadoras FasL (ligante de FAS), TNF (fator de necrose tumoral) e TRAIL (ligante indutor de apoptose relacionado ao TNF) que se ligam a receptores de morte da superfície celular, desencadeando a clivagem e ativação da proteína caspase-8, que induz a ativação de caspases efetoras do processo apoptótico e clivagem de substratos (Elmore, 2007).

A via intrínseca (ou mitocondrial) da apoptose, ativada quando a célula sinaliza algum dano de DNA, estresse do retículo endoplasmático ou estresse oxidativo, é modulada por uma família de proteínas, a família Bcl-2. Esta família é dividida entre antiapoptóticas (Bcl-2, Bcl-XL, Bcl-W, MCl-1 e BFL-1) e pró-apoptóticas (BH3, Bik, Blk, BNIP-3, Bim-L, Bad, Bid, PUMA, NOXA, Bax e Bak) (Martinou & Youle, 2011). Em linhas gerais estas proteínas modulam a permeabilidade da membrana mitocondrial (Adams & Cory, 1998; Cory & Adams, 2002). Como pode ser observado na figura 1, após o sinal de ativação ocorre um desbalanço entre proteínas pró e antiapoptóticas levando a formação de poros na membrana mitocondrial e a liberação de citocromo c para o citoplasma. A liberação de componentes intramitocondriais para o espaço citosólico ativa Apaf-1, formando o apoptossomo que cliva e ativa a caspase-9, que por sua vez ativa as caspases 3, 6 e 7, executoras do processo apoptótico (Elmore, 2007; Riedl et al., 2004).

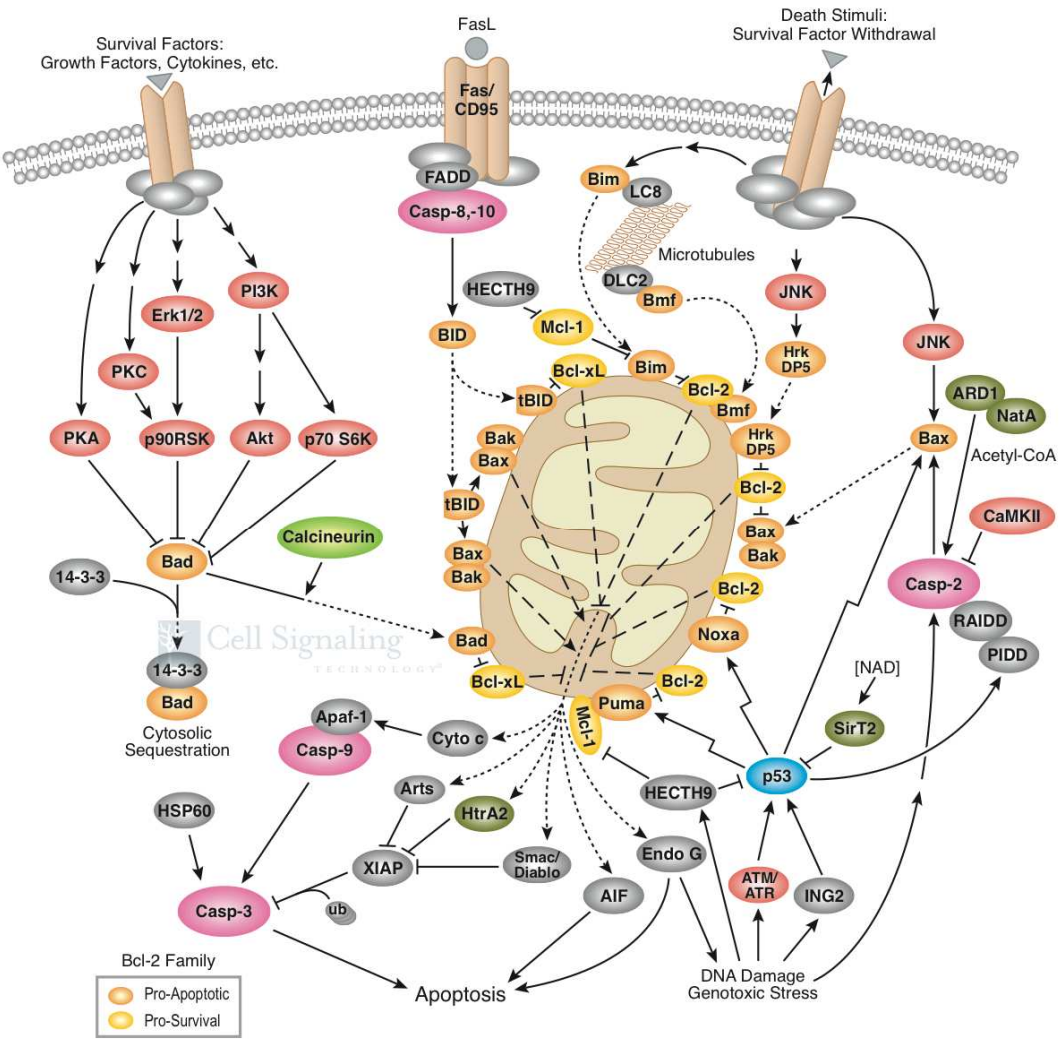

Figura 1: Controle mitocondrial da apoptose. As cores apresentadas não têm relevância para o entendimento geral da via de sinalização. Setas indicam ativação direta e sinais fechados representam inibição. Obtido de http://media.cellsignal.com/www/pdfs/science/pathways/Apoptosis_Mitochondrial.pdf. (Acessado em 23/10/2014).

As Neks e a morte celular

O protótipo da família das Neks, NIMA, identificado em *A. nidulans*, é uma proteína essencial para a correta transmissão de cromossomos para células filhas. Defeitos ou ausência desta proteína provocam a parada do ciclo celular em G2/M (Osmani et al., 1991; Lu & Hunter, 1995) ou caso a célula consiga progredir no ciclo

podem ser observados defeitos na formação do fuso mitótico (Lu & Hunter, 1995) ou até mesmo apoptose induzida por mitose catastrófica (Löffler et al., 2006). Uma importante etapa do processo apoptótico é a permeabilização da membrana mitocondrial após um dano citotóxico ou genotóxico (Wang, 2001). A proteína VDAC é uma importante mediadora deste processo que, em condições normais, além de regular o fluxo de ATP pelo fechamento dos poros, também impede a liberação do citocromo c para o citoplasma principalmente através da ruptura da membrana mitocondrial externa (Rostovtseva, 2008). A abertura e fechamento do poro de permeabilidade de transição é regulada pela interação direta entre VDAC e Nek1 através da fosforilação de VDAC na serina 193 por Nek1 , prevenindo a morte celular excessiva após injúria (Chen et al., 2009; Chen et al., 2010). Experimentos em células kat2J, células de camundongo contendo Nek1 inativa, apresentam altas taxas de apoptose (Chen et al., 2014). Pelegrini e colaboradores (2010) demonstraram que após indução de dano de DNA com diferentes drogas, mutantes de Nek1 (*knockdown* - KD) não apresentaram bloqueio em G2/M, gerando dessa forma, células com defeitos mitóticos. Assim, Nek1 também está envolvida no começo da resposta a estresse genotóxico e possui um papel importante na prevenção de morte celular induzida por danos de DNA (Pelegrini et al., 2010).

Diversos outros membros da família das Neks tem sido relacionados direta ou indiretamente ao processo de morte celular. Experimentos de microarranjo de DNA demonstraram que o tratamento com estaurosporina induz alterações na expressão de Nek2 em células NIH3T3 (Treviño et al., 2014). Similarmente, tratamento com TAI-95 em câncer de fígado promovem a degradação de Nek2 levando as células à defeitos no alinhamento de cromossomos e morte celular excessiva (Huang et al., 2014), assim como no tratamento de células de câncer de mama silenciadas para Nek2 e tratadas com paclitaxel e doxorrubicina (Lee & Gollahon, 2013). A superexpressão de Nek2 em células de câncer resultou em instabilidade cromossômica aumentada, proliferação celular e resistência a drogas aumentada; ao passo que o silenciamento por shRNA aumentou a sensibilidade a drogas e induziu apoptose *in vitro* e em modelos de mieloma em camundongos (Zhou et al., 2013). O papel de Nek3 no processo apoptótico parece ser semelhante a Nek2, a superexpressão de Nek3 inativa (*kinase dead*) em células T47D levaram ao aumento de 50% no níveis de células apoptóticas (Miller et al. 2005).

A indução de senescência em células de câncer foi proposto como uma efetiva estratégia de tratamento do câncer e, alterações nos níveis de expressão de proteínas envolvidas neste processo se tornam promissoras. A inibição de Nek6 por siRNA ou a superexpressão de Nek6-KD provocam entrada prematura na senescência, assim como, elevadas taxas de apoptose em vários tipo celulares de câncer (Jee et al., 2013; Nassirpour, 2010). O'Regan e colaboradores (2009) demonstraram que os efeitos de Nek6-KD ou com expressão reduzida por siRNA têm efeito semelhante ao silenciamento de Nek7 ou expressão da versão inativa no sentido de ambas promoverem apoptose e defeitos na progressão mitótica.

O *checkpoint* de dano de DNA previne que as células entrem em divisão enquanto as lesões de DNA estiverem presentes através da degradação de CDC25A, um ativador de CDKs. Para que ocorra esta degradação é importante a fosforilação de CDC25A por Nek11. Inibição da atividade de Nek11 promove um bloqueio das células na mitose e posterior morte celular. Adicionalmente, a expressão de Nek11 está aumenta durante o desenvolvimento de câncer de cólon (Sørensen et al., 2010).

Poliglutamilação

O citoesqueleto de microtúbulos é essencial para a organização interna de células de eucariotos (Bieling et al., 2008). Microtúbulos são estruturas filamentares com uma variedade de funções em células vivas, por exemplo, na divisão celular, diferenciação neuronal e transporte no interior das células. Eles são formados a partir da polimerização ordenada de alfa e beta tubulinas. A grande diversidade de tubulinas dentro de uma célula é devida à expressão de diferentes isotipos de tubulina e um grande número de modificações pós-traducionais (PMT), como acetilação/desacetilação, remoção e adição de tirosina na região C-terminal, fosforilação, poliglicilação e poliglutamilação. A glutamilação e glicilação são dois tipos de modificação pós traducional que foram inicialmente descobertas em tubulinas (Eddé et al., 1990; Redeker et al., 1994) e consistem na adição de cadeias laterais de glutamato adicionadas às proteínas. Esta modificação está presente em vários tipos de tubulinas, ao passo que a glicilação é restrita

a axonemas de cílios motores e flagelos (Bré et al., 1996; Plessmann & Weber, 1997; Iftode et al., 2000) (Figura 2).

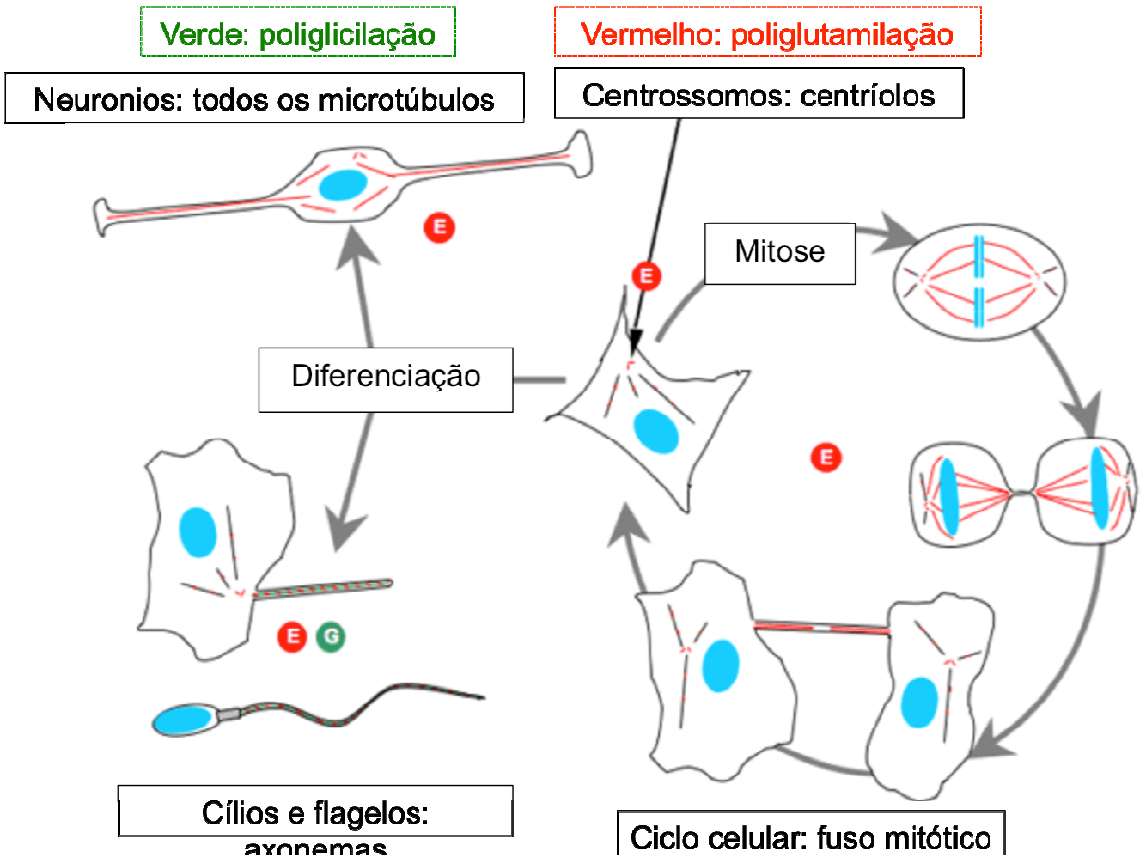

Figura 2: A poliglutamilação pode ocorrer em diferentes substratos. Microtúbulos são altamente modificados em cílios e flagelos, centríolos durante o ciclo celular e em neurônios. Adaptado de https://perso.curie.fr/Carsten.Janke/ (acessado em:15/02/2015).

A glutamilação é encontrada em microtúbulos de cílios e flagelos (Lechtreck & Geimer, 2000), centríolos, centrossomos e corpos basais (Bobinnec et al., 1998b) (Figura 2). Em neurônios a maioria dos microtúbulos são modificados pela adição de 1 a 6 resíduos de glutamato (Audebert et al., 1994). Células em proliferação e/ou interfáse apresentam baixos níveis de glutamilação, porém, durante a divisão, microtúbulos do fuso mitótico apresentam níveis aumentados de tubulinas glutamiladas (Regnard et al., 1999; Verhey & Gaertig, 2007) (Figura 3).

Populações de microtúbulos podem ser distinguidas entre diferentes tipos celulares, assim como dentro de uma célula específica através dos níveis de glutamilação. Estes microtúbulos podem ser diferenciados de acordo com: I- a densidade das modificações; II- o tipo de tubulina modificada (α e β tubulinas); III- o tamanho da cadeia de poliglutamilação e IV- o local de inserção das cadeias de glutamato na molécula de tubulinas. Os níveis de poliglutamilação podem ser responsáveis por um "código" das tubulinas e dessa forma regular sua polimerização e/ou alteração com proteínas motoras.

A maioria das modificações de tubulinas apresentam importantes papéis na função de microtúbulos (revisado por: Luduena, 1998; Westermann & Weber, 2003; Verhey & Gaertig, 2007). A glutamilação ocorre no domínio C-terminal de tubulinas (Eddé et al., 1990; Redeker et al., 1994), que são sítios importantes para a interação com proteínas associadas a microtúbulos (*microtubule-asociated proteins – MAPs*) e proteínas motoras baseadas em microtúbulos, como as cinesinas e dineínas. Dessa forma, acredita-se que a poliglutamilação possa desempenhar um papel importante na interação de microtúbulos e seus parceiros (Ikegami et al., 2007, Nogales, 2000). Experimentos *in vitro* demonstraram que o estado de poliglutamilação de α ou β tubulinas dos microtúbulos atuam como regulador da interação de tubulinas com MAPs e cinesinas (Boucher et al., 1994; Larcher et al., 1996; Bonnet et al., 2001). Muitos outros processos dependentes de microtúbulos como a motilidade de cílios e flagelos (Cosson et al., 1996; Fouquet et al., 1994, Gagnon et al., 1996, Million et al. 1999), a estabilidade de centríolos (Bobinnec et al., 1998a e b; Regnard et al., 2003; Abal et al., 2005), controle do ciclo celular (Bobinnec et al., 1998b; Regnard et al., 1999) e crescimento e estabilidade de neurônios (Ikegami et al., 2006, Audebert et al., 1993; 1994; Boucher et al., 1994; Larcher et al., 1996; Bonnet et al., 2001)) parecem ser controlados pela poliglutamilação (Figura 3).

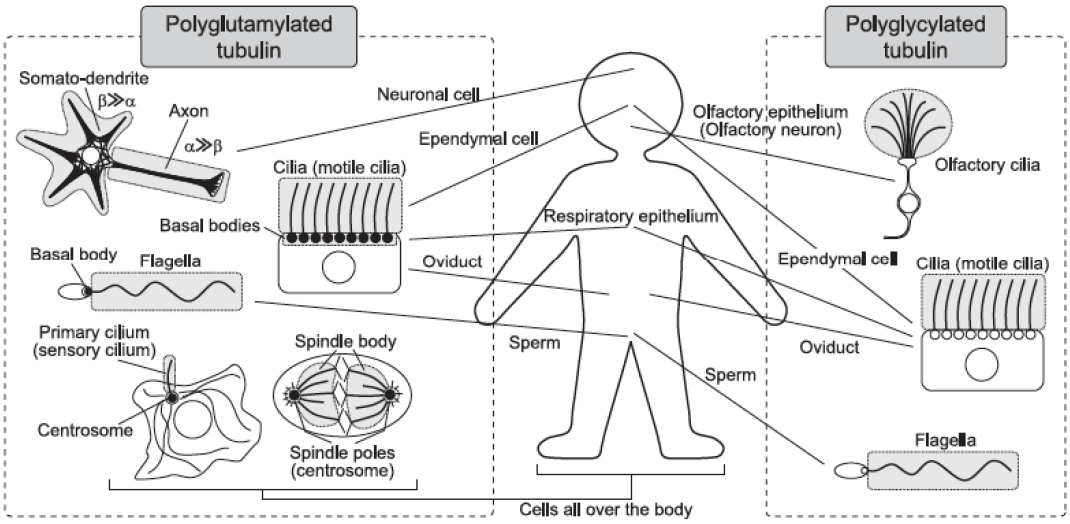

Figura 3: Distribuição de tubulinas poliglutamiladas e poligliciladas. As subunidades de tubulinas que estão poliglutamiladas são diferentes entre somato- dendrito e axônios. Atualmente nenhuma evidencia está disponível sobre a poliglicilação de tubulinas em centríolos. Adaptado de Ikegami & Setou, 2010.

Duas outras modificações pós-traducionais de tubulinas, a detirosinação (Barra et al., 1988) e geração de Δ2-tubulina (Paturle-Lafanechere et al., 1994), também ocorrem na região C-terminal de α tubulinas especificamente. Ambas as modificações são funcionalmente ligadas a poliglutamilação (Banerjee, 2002). A detirosinação é a remoção reversível de tirosinas do C-terminal gerando uma tubulina detyr e expondo o resíduo Glu subsequente. A Δ2-tubulina é a remoção irreversível deste resíduo –glu, gerando portanto, uma tubulina chamada Δ2-tubulina. A detirosinação tem sido observada em diferentes espécies de microtúbulos e é normalmente encontrada em microtúbulos estáveis e de longa vida. A função da detirosinação é a regulação da interação microtúbulos-MAP cinases. As proteínas CAP-Gly, um subgrupo de proteínas de tráfego *plus-end* (+TIPs; Akhmanova & Steinmetz, 2008), são reguladas pelo estado de tirosinação de α-tubulinas (Peris et al., 2006, Bieling et al., 2008). Δ2-tubulinas por sua vez, são especificamente localizadas em neurônios e axonemas, onde microtúbulos são altamente glutamilados (Lafanechere & Job, 2000), entretanto, suas funções ainda não são claras.

A *tubulin tyrosine ligase* (TTL) foi a primeira enzimas descrita como envolvida na modificação de tubulinas (Ersfeld et al., 1993) e outras enzimas envolvidas neste

processo foram identificadas recentemente (Tabela 2). Ensaios de imunoprecipitação de microtúbulos de neurônios permitiram a purificação de tubulinas complexadas com a proteína *tubulin tyrosine ligase like 1* (TTLL1) (Janke et al., 2005). A sequência de similaridade entre TTLL1 e TTL sugere que a maioria das enzimas que modificam a região C-terminal de tubulinas pela ligação de aminoácidos pertencem a família das TTLs. Atualmente, em mamíferos, foram identificados 12 membros para a família das TTLLs, 9 glutamilases e 3 glicilases (Rogowski et al., 2009). Os principais processos biológicos, assim como funções prospostas e doenças associadas aos demais membros desta família de proteínas podem ser visualizados na tabela 2.

Estudos da atividade catalíticas destas enzimas revelaram que a heterogeneidade de polimodificações de microtúbulos se devem a especificidade de substratos destas enzimas. As poliglutamilases são caracterizadas pela preferência pela iniciação ou elongamento das cadeias glu, ou pela seletividade a α ou β tubulina, ou ainda por outros substratos. Na tabela 2 é possível observar a preferência de substrato de diferentes membros da família, assim como, o papel desempenhado na iniciação ou elongamento da cadeia de glutamilação (Figura 4). Como várias enzimas localizam em determinados subtipos de microtúbulos dentro da célula e a disponibilidade de enzimas possuem expressão tecido-específica, sugere-se que exista um mecanismo multi-enzimático que regula as vias de poliglutamilação (van Dijk et al., 2007, Rogowski et al., 2009, review: Janke et al., 2008).

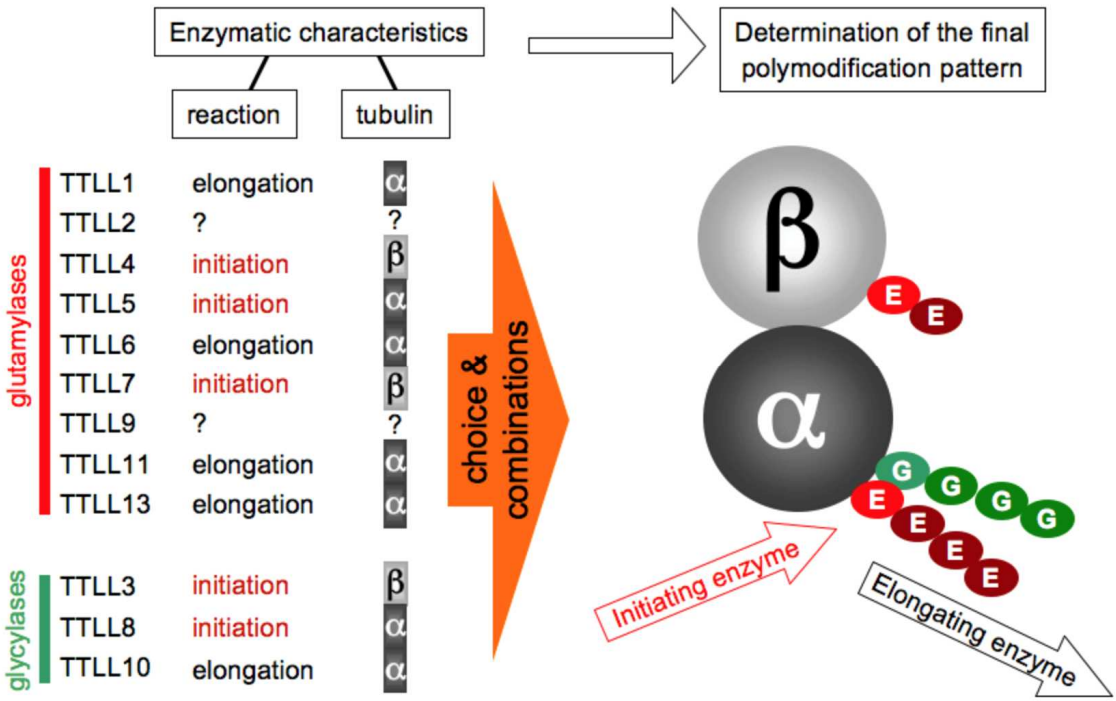

Figura 4: Preferência de reação de glutamilases e glicilases. Iniciação: a enzima tem alta atividade na adição do primeiro resíduo da cadeia; elongação: a enzima tem uma atividade de elongamento da cadeia lateral; preferência por α- ou β-tubulina estão indicadas). Diferentes vias de modificações são geradas pela combinação de diferentes enzimas. (Janke et al., 2005; van Dijk et al., 2007, Rogowski et al., 2009, review: Janke et al., 2008).

Apesar de estar associada principalmente a tubulinas, a poliglutamilação não é restrita a tubulinas. Foi demonstrado que ocorrem em proteínas de montagem de nucleossomos, NAP1 e NAP2 (Regnard et al., 2000), e, provavelmente, em várias proteínas ainda não identificadas. Proteínas ricas em glutamina poderiam ter um "papel natural" na indução de morte celular desregulada em doenças neurodegenerativas (Blum et al., 2012). TTLL4 pertence a uma família de proteínas com um domínio de homologia TTL e pode catalisar a ligação de diversos aminoácidos através dos processos de tirosinação, poliglicilação, e poliglutamilação em vários substratos.

As Neks compreendem uma família de proteínas que regulam eventos mitóticos incluindo a entrada na mitose, separação de centrossomos, formação do fuso e citocinese (O'Connell et al., 2003; O'Regan et al., 2007). Westermann et al., (2003) detectaram a primeira evidência do envolvimento de Nek em *Crithidia fasciculata* no processo de

poliglutamilação. Através de cromatrografia utilizando hidroxiapatita como passo final, tubulinas poliglutamiladas copurificaram juntamente com p54, um membro da família das Neks em *C. fasciculata.* A expressão de p54 com cauda His permitiu a identificação desta proteína no corpo basal e flagelo deste parasita, regiões sabidamente altamente glutamiladas. Eles demostraram ainda, que o extrato purificado de p54 foi capaz de poliglutamilar tubulinas *in vitro*. Além disso, diversas funções estão interligadas entre Nek e TTLLs, como por exemplo a localização de muitos membros da família das Neks em regiões altamente glutamiladas, como centrossomos, *midbodies*, fuso mitótico, dentre outros.

Tabela 2: Funções propostas para a família das TTLLs. O tamanho, domínio TTL, processos biológicos, função e doenças associadas de cada um dos membros da família das TTLLs estão apresentados na tabela.

Proteína	Nome	Nomes alternativos	Tamanho (a.a./kDa)	Domínio TTL	Processos Biológicos	Função	Doenças associadas
TTLL1	Tubulin--tyrosine ligase-like protein 1	Tubulin polyglutamylase complex subunit 3, PGs3	423 / 49	1–367	Montagem do axonema ciliar, movimento ciliar, poliglutamilação	Subunidade catalítica do complexo poliglutamilase de tubulinas neuronais[1, 2]; modificaçõ de alfa e beta tubulinas[1].	Hipertrofia cardíaca[3]; Sinusite, rinosinusite, otite e infertilidade masculina em camundongos[4]
TTLL2	Tubulin-tyrosine ligase-like protein 2	Testis-specific protein NYD-TSPG	592 / 67	84–427	Poliglutamilação	Atua em complexo com outras proteínas.	
TTLL3	Tubulin-tyrosine ligase-like protein 3	HOTTL	772 / 87	151–510	Montagem do axonema, movimento ciliar, glicilação	Monogicilases de alfa e beta tubulinas[5, 2], iniciação da reação de glicilação[2].	Desenvolvimento de carcinoma coloretais[6]; Desenvolvimento embrionário[9]
TTLL4	Tubulin-tyrosine ligase-like protein 4		1199 / 133	604–947	Poliglutamilação	Modifica preferencialmente beta-tubulinas e proteínas de montagem do nucleossomo, NAP1 e NAP2[7]; Iniciação da reação de glutamlação[7, 8].	Megacariopoiese[10]; Adenorcarcinoma pancreático ductal[11]
TTLL5	Tubulin-tyrosine ligase-like protein 5	SRC1 and TIF2-associated modulatory protein,	1281 /144	62–407	Poliglutamilação, transcição dependente de DNA	Modifica preferencialmente alfa-tubulinas[7, 8]; Iniciação da reação de glutamilação[7, 8] Requerida para a localização de CSAP em microtúbulos do fuso e dos cílios[12]; Repressão de receptores glucocorticóides e indução de receptores adrógenos[13].	Degeneração da retina[14]; Infertilidade masculina[13]; Câncer do endométrio[15]
TTLL6	Tubulin-tyrosine ligase-like protein 6		843 / 96,4	58–401	Fomação e separação de microtúbulos, poliglutamilação	Modifica preferencialmente alfa-tubulinas[7, 8]; Elongamento da reação de glutamilação[7, 8]; Medeia poliglutamilação de	Megacariopoiese[10]; Deficiência da motilidade ciliar[27]; Alzheimer[28]; Síndrome

						tubulinas ciliares[16, 21].	de Joubert[29]
TTLL7	Tubulin-tyrosine ligase-like protein 7	Testis development protein NYD-SP30	887 / 103	38–390	Diferenciação, desenvolvimento do sistema nervoso, poliglutamilação	Modifica preferencialmente beta-tubulinas[7, 8]; Iniciação da reação de glutamilação[7, 8]; Necessária para o crescimento de neuritos[17].	Câncer[18]; Obesidade[19]; Desenvolvimento neuronal[20]
TTLL8	Tubulin-tyrosine ligase-like protein 8		850 / 94,7	222–580	Glicilação	Modifica preferencialmente alfa tubulinas e outras proteínas como ANP32A, ANP32B, SET e NCL; Iniciaçãoda glicilação[21].	Defeitos em cílio ependimais[21]
TTLL9	Tubulin-tyrosine ligase-like protein 9		439 / 51,5	40–380	Poliglutamilação	Atua em complexo com outras proteínas no processo de poliglutamilação.	
TTLL10	Tubulin-tyrosine ligase-like protein 10		673 / 75	155–552	Glicilação[22]	Poliglutamila tubulinas quanto co-expressa com TTLL8[23]	
TTLL11	Tubulin-tyrosine ligase-like protein 11		538 / 58	218–538	Poliglutamilação	Modifica preferencialmente alfa-tubulinas[7, 8]; Elongamento da reação de glutamilação[7, 8]; Necessária para a localização de CSAP nos microtúbulos do fuso e dos cílios[12].	Esquizofrenia[24]
TTLL12	Tubulin--tyrosine ligase-like protein 12		644 / 74,4	300–644	Poliglutamilação	-	Câncer de próstata[25, 26]
TTLL13	Tubulin polyglutamylase TTLL13	Tubulin--tyrosine ligase-like protein 13	815 / 93,6	85–430	Poliglutamilação	Modifica preferencialmente alfa-tubulinas[7, 8]; Elongamento da reação de glutamilação[7, 8]	

1-Janke et al., 2005; 2- Pathak et al., 2011; 3- Song et al., 2012; 4- Vogel et al., 2010; 5- Wloga et al., 2009; 6- Rocha et al., 2014; 7- van Dijk et al., 2007; 8- Regnard et al., 1999; 9- Cochran et al., 2013; 10- Ye et al., 2014; 11- Kashiwaya et al., 2010; 12- Backer et al., 2012; 13- Lee et al., 2013; 14- Sergouniotis et al., 2014; 15- Liang et al., 2012; 16- Pathak et al., 2007; 17- Ikegami et al., 2007; 18- Das et al., 2014; 19- Lee et al., 2011; 20- Ikegami et al., 2006; 21- Bocsh et al., 2013; 22- Ikegami et al., 2008; 23- Ikegami et al., 2009; 24- Fullston et al., 2011; 25- Massoner et al., 2012; 26- Wasyllyk et al., 2010; 27- Suryavanshi et al., 2011; 28- Zempel et al., 2013; 29- Lee et al., 2012.

Objetivos

Objetivo Geral

Este trabalho teve como objetivo geral a compreensão de algumas funções exercidas pela proteína Nek5 no contexto celular através da análise de suas interações com outras proteínas.

Objetivos Específicos

- Identificar as proteínas e/ou substratos que interagem intracelularmente com a Nek5 humana bem como caracterizar sua interação.
- Caracterizar o papel de Nek5 no processo de morte celular e defeitos na cadeia respiratória (Artigo I).
- Caracterizar a interação de Nek5 com a proteína TTLL4 (Artigo II).
- Avaliar o papel de Nek5 no processo de poliglutamilação (Artigo II).
- Caracterização estrutural de Nek1.

Resultados

Artigo I

"Stop Ne(c)king around": How interactomics contributes to functional characterize Nek family kinases

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REVIEW

"Stop Ne(c)king around": How interactomics contributes to functionally characterize Nek family kinases

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Author contributions: Meirelles GV, Perez AM, de Souza EE, Basei FL, Papa PF, Melo Hanchuk TD, Cardoso VB, Kobarg J performed the literature search, analysis and interpretation of the data, and contributed specific parts of the manuscript; Meirelles GV and Kobarg J elaborated the figures; Kobarg J, Meirelles GV and Perez AM conceived the overall idea of the review, elaborated the final version of the text together, and supervised the project; all the authors read, revised and approved the final version.

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Abstract

Aside from Polo and Aurora, a third but less studied kinase family involved in mitosis regulation is the never in mitosis-gene A (NIMA)-related kinases (Neks). The founding member of this family is the sole member NIMA of Aspergillus nidulans, which is crucial for the initiation of mitosis in that organism. All 11 human Neks have been functionally assigned to one of the three core functions established for this family in mammals: (1) centrioles/mitosis; (2) primary ciliary function/ciliopathies; and (3) DNA damage response (DDR). Recent findings, especially on Nek 1 and 8, showed however, that several Neks participate in parallel in at least two of these contexts: primary ciliary function and DDR. In the core section of this in-depth review, we report the current detailed functional knowledge on each of the 11 Neks. In the discussion, we return to the cross-connections among Neks and point out how our and other groups' functional and interactomics studies revealed that most Neks interact with protein partners associated with two if not all three of the functional contexts. We then raise the hypothesis that Neks may be the connecting regulatory elements that allow the cell to fine tune and synchronize the cellular events associated with these three core functions. The new and exciting findings on the Nek family open new perspectives and should allow the Neks to finally claim the attention they deserve in the field of kinases and cell cycle biology.

Key words: Cell cycle; Mitosis; DNA damage response; Protein interactions; Kinases

Core tip: Never in mitosis-gene A (NIMA)-related kinases (Neks) are a family of 11 human kinases involved in cell cycle regulation. This article represents an in-depth review of the current knowledge on the function of each of the 11 human Nek kinases. Furthermore, we present arguments in the discussion of how systems biology, especially interactomics, helped to uncover that the majority of Neks are involved in more than one of

the three Neks core functions: (1) centrioles/mitosis; (2) primary ciliary function/ciliopathies; and (3) the DNA damage response. Possibly, the Neks act on a higher regulatory level which may control the core functions.

Meirelles GV, Perez AM, de Souza EE, Basei FL, Papa PF, Melo Hanchuk TD, Cardoso VB, Kobarg J. "Stop Ne(c)king around": How interactomics contributes to functionally characterize Nek family kinases. *World J Biol Chem* 2014; 5(2): 141-160 Available from: URL: http://www.wjgnet.com/1949-8454/full/v5/i2/141.htm DOI: http://dx.doi.org/10.4331/wjbc.v5.i2.141

INTRODUCTION

The never in mitosis-gene A (NIMA)-related kinases (Neks) represent, aside from the Polo and Aurora kinase families, a third family of mitotic kinases, but remain the least studied to date and hence least understood family of kinases involved in the regulation of the cell cycle. The founding member of this family of kinases is the *Aspergillus nidulans* NIMA, which exists as a single member in this fungus, is functionally involved in the initiation of mitosis and promotes the chromosome condensation by phosphorylation of histone H3[1]. Humans have 11 members of the Nek family which show highly conserved kinase domains but differ significantly in the composition and length of their N- and especially C-terminal regulatory and docking domains (Figure 1).

Although some protein interaction partners have been described for the majority of the human Neks (Figure 2), the domain of interaction at the side of Neks has been mapped only for a smaller subset of interacting proteins (Figure 1). As we can see, most interactors are assigned to specific regions in the regulatory domains, which represent in most cases classical protein-protein interaction modules, such as coiled coil regions. Identification of interaction with the kinases domains have been scarce due to the transient and weak nature of these interactions and therefore the discovery and characterization of true *bona fide in vivo* substrates of Nek kinases remain one of the main challenges in the field. Among the interacting proteins identified by our[2,3] and other groups, through both yeast two-hybrid screens and mass spectrometry analyses, there were hopefully not only those that regulate the Neks but maybe also candidate substrate proteins. The binding of these substrate proteins possibly contributes to "opening up" the Neks or to the activation of these kinases and then, as a consequence, these proteins may be phosphorylated by the Neks.

There has been a series of very good and concise reviews on NIMA and Neks in the past years[4-8]. However, due to scarce or absent knowledge on several family members, including Nek5, 10 and 11 for instance, most reviews opted to focus on a subset of Neks or grouped them according to phylogenetic or functional relatedness. Here, we try to discuss all 11 human Neks in some depth and to include all recent novelty on the least studied Neks as well as our own group's published and unpublished findings, with a special emphasis on the characterization of the functional context based on the identification of interacting proteins (interactomics). A point we would like to stress here is that most Neks interact with proteins of several of the classical functional contexts reported initially for a subset of specific Neks. In other words, we may characterize the following three areas as the main functional contexts of Neks: (1) centriolar function and mitosis regulation (Nek2, 6, 7 and 9); (2) primary ciliary function, ciliopathies and microtubule dynamics in general (Nek1, 4 and 8); and more recently (3) DDR and G_2/M checkpoint (Nek1, 4, 6, 8, 10 and 11)[8,9].

However, published interactome data (Figure 2), as well as our group's efforts to identify new interacting proteins for all Neks, showed some surprising cross-connections and novelties, which we would like to point out here. Most of the above mentioned Neks seem to interact with proteins that are functionally linked to two or even all three of the above mentioned areas, thereby raising the possibility that these are somehow connected on a higher regulatory level and that the Neks may be key elements to understand how the regulation of these functional contexts is performed. A typical recently published example is the role of Nek8 in both primary ciliary function and DNA repair mechanisms[10]. Our own studies revealed that Nek6, a kinase primarily associated with mitotic regulatory events[11,12], also interacts with proteins involved in the DNA damage response, such as putative DNA repair and recombination protein RAD26-like (RAD26L) and PHD finger protein 1 (PHF1) (Figure 2)[3]. In fact, for the majority of Neks we found interacting partners of the DDR or effector proteins of different DNA repair pathways, which clearly suggests a larger than initially imagined involvement of Neks in these biological processes. Other insights came from the identification of interacting proteins from the apoptosis regulatory pathways with several Neks (*e.g.*, Nek 1[13] and 5). This suggests that, aside the well established mitotic context, we must be open minded about additional new roles for Neks (Table 1). Before we go into details of new cross-connections and suggested additional functional contexts in the final discussion, we will present each of the 11 human Neks in detail in the following section of this review.

NEK1

Although Nek1 is only the third most studied Nek family member after Nek2 and aside from Nek6, it is in many ways a representative member of this family of protein kinases. Along this line, Nek1 started to draw the attention of the kinase and signaling research communities, not only to itself but to the Nek family after the publication of the seminal article of Upadhya *et al* in 2000[14]. It reported that deletion mutations in the Nek1 gene in mice caused polycystic kidney disease (PKD) among other pleiotropic effects, ranging from facial dysmorphism,

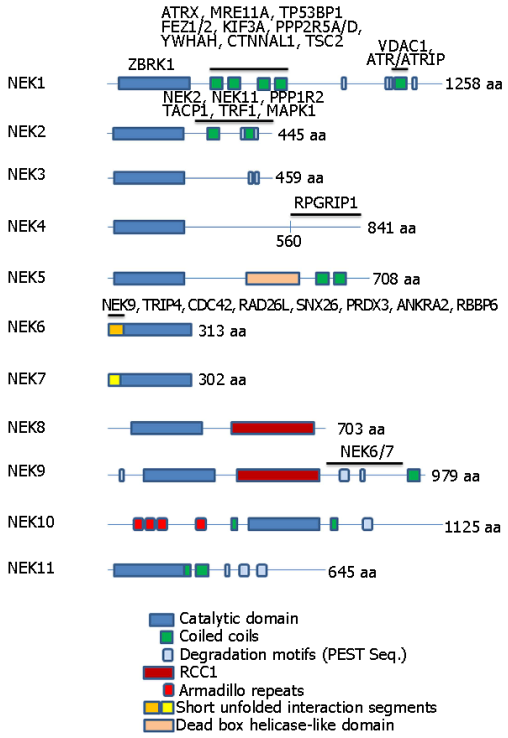

Figure 1 Representation of the domain organization of the eleven human Neks depicting the domain regions for selected protein interactions. The gene symbols corresponding to interacting proteins are shown above the Neks primary structure regions with which they have been found to interact. The list of interactors is not intended to be complete but is necessarily shorter than the list of all proteins known in the literature to interact with Neks (*e.g.*, see Figure 2), since, for the majority of interactors, the location of interaction in the Neks has not been reported. Different repeated domains have been indicated by the color code at the bottom of the figure. The lengths of the full proteins are indicated by number of amino acids (aa) at the C-terminal of the proteins. At least two isoforms of Nek1, 2, 3 and three of Nek4 and 11, all generated by alternative splicing, have been reported and known functional distinctions have been briefly discussed in the text, where feasible. References for the proteins and their mapped interactors: Nek1[2,13,25]; Nek2[116,121-124]; Nek4[53]; Nek6[3]; Nek9[66]. Nek: Never in mitosis-gene A-related kinases.

dwarfing, male sterility, anemia and cystic choroid plexus. The pleiotropic nature of these phenotypes suggested a role of Nek1 early on in basic cellular functions, possibly involved in signaling pathways associated with polycystin-1 and 2, whose mutations also cause PKD and signaling initiates at the renal epithelial cell's primary cilia[15].

Recently, another set of insertion, non-sense and splice site mutations in the Nek1 gene were reported in Majewski type short-rib polydactyl syndrome (SRPS), an autosomal-recessive familiar ciliopathy[16,17]. Ciliopathies have been associated with a series of defects of proteins involved in intra-flagellar transport (IFT), as well as cilia, basal body and centrosome maintenance, and in the case of Nek1, SRPS also presents a broad phenotypic spectrum, including reduced cilia number and cell cycle associated cilia morphogenesis. This results ultimately in severe or lethal embryonic malformations and especially osteochondrodysplasia, shortened ribs and tibias, polysyndactyly, fused kidneys, heart defects and mouth clefts, among others[17].

In terms of molecular functions, a first breakthrough came from a protein interactome study that shed light on the involvement of Nek1 in several pathways related to the above diseases, but also opened new avenues in the context of cell cycle regulation and DNA damage responses[2]. These findings were later not only confirmed by functional studies but also extended to other Nek family members, including Nek4, 6, 10 and 11[3,8,9,18]. The interactome study was a yeast two-hybrid assay using Nek1 as bait and a human fetal brain cDNA library as prey. Nek1 is a rather large, 1258 amino acids containing protein and interacts with these proteins mainly through the two N-terminals of its four coiled coil regions, which are located at the C-terminal of its kinase domain (Figure 1). Among the Nek1 interacting proteins were the kinesin-like protein KIF3A, tuberin and alpha-catulin, mutation in all three of these genes also have been reported to cause PKD. This suggests the existence of a multicomponent signaling or regulatory pathway, which regulates the kidney cell's proliferation and when affected by mutations may lead to PKD[19-21]. Evidence in support for a major role of Nek1 in primary ciliary function also came from other model organisms, including *Chlamydomonas*[22].

Surprising at that time was the discovery of interactions with several cell cycle regulatory proteins, 14-3-3 protein η (*eta*, YWHAH), tumor suppressor p53-binding protein 1 (TP53BP1), serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit alpha/delta isoform (PPP2R5A/D) and especially with proteins involved in the DNA damage response, such as the double-strand break repair protein MRE11A (MRE11A) and the transcriptional regulator ATRX (ATRX)[2]. Soon, additional experiments with the irradiation of wild-type and Nek1-/- cells revealed that Nek1 is overexpressed and activated in response to ionizing radiation (IR) and co-localizes to γ-H2AX positive DNA repair foci in the nucleus[23]. Cells without Nek1 died in response to sub-lethal doses of IR and knockdown of Nek1 also diminished their capacity to clear DNA damage caused by chemical genotoxic agents, such as cisplatin and methyl-metanesulfonate (MMS)[24]. This line of experiments culminated recently in a paper where the authors showed that Nek1 kinase is not only physically associated with ATR-ATRIP, but also required for ATR priming to allow an efficient DNA damage signaling[25]. Furthermore, Nek1 has been indicated to act in apoptosis signaling, especially by phosphorylation of key mitochondrial proteins such as the voltage-dependent anion-selective channel protein 1 (VDAC1)[13]. This is a pore complex that functions both as a voltage dependent anion channel and permeability pore that regulates cytochrome c leakage to the cytoplasm, which upon exit initiates apoptotic events[13]. Nek1's activity to maintain cells in homeostasis is mediated through phosphorylation of a specific external VDAC1 Ser residue. Upon apoptotic stimuli, Nek1 is degraded and the lack of

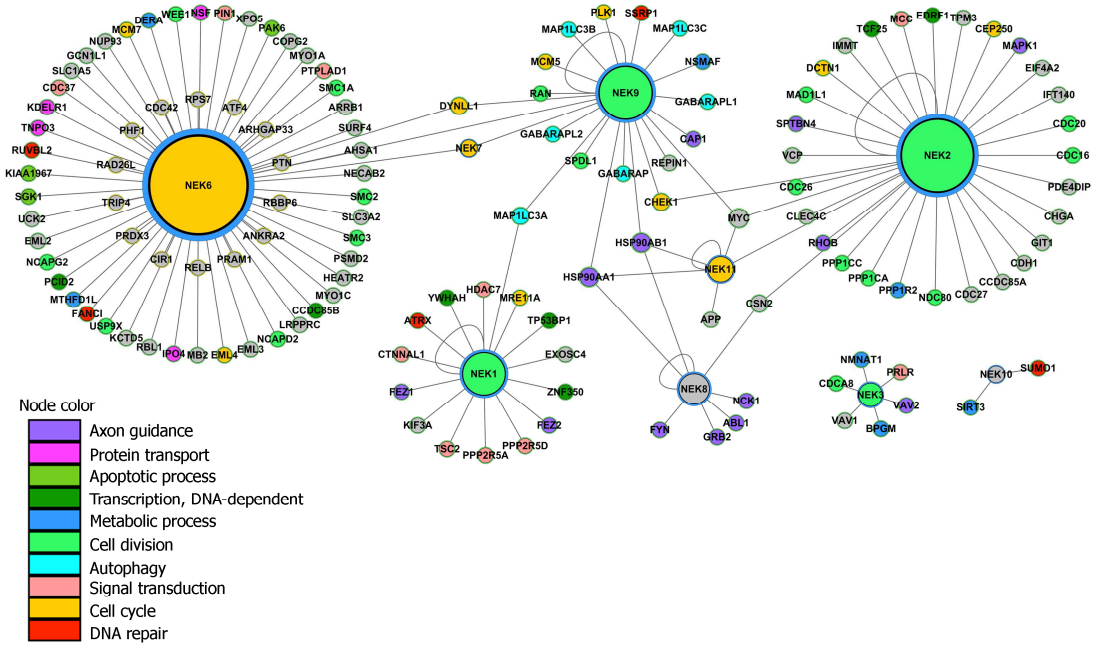

Figure 2 Global interactome of Nek1-11, involving their published interactors. The proteins color code refers to their main biological function given by the top enriched Gene Ontology[125] biological processes ($P \leqslant 0.05$). Common interactors establish crosslinks between Neks, thereby emphasizing their common functional contexts. The protein sizes are depicted proportional to their connectivity degree. The protein-protein interaction network was built for the first neighbors of Neks using the Integrated Interactome System (IIS) platform, developed at National Laboratory of Biosciences, Brazil (http://www.lge.ibi.unicamp.br/lnbio/IIS/) and visualized using the Cytoscape software[126]. Nek: Never in mitosis-gene A-related kinases.

VDAC1 phosphorylation causes opening of the channel, loss of the membrane potential and leakage of cytochrome c to the cytoplasm.

Finally, Nek1 has been implicated in gametogenesis due to its high expression levels in meiotic tissues[26]. In another interactome study, this time using a testicular tissue cDNA library, the protein Nurit was found to be an interactor of Nek1[27]. Nurit is expressed in the late phase of spermatogenesis, has structural resemblance with leucin zippers and contains additional super helix domains, possibly involved in its homo-multimerization. Furthermore, the structural maintenance of chromosomes protein 3 (SMC3) was found to interact with Nek1, further implying important functions in meiotic events such as spindle assembly checkpoints[28].

In summary, Nek1 has been functionally implied in three major functional contexts and their sub-functions: ciliogenesis (PKD, SRPS), DNA damage response in a wider sense, also including cell cycle checkpoints and centrosome functions and, finally, gametogenesis. Unpublished recent mass spectrometry studies of the Nek1 interactome after challenging cells with genotoxic drugs identified a number of nuclear proteins, the majority of which were associated with DNA repair, replication and transcription regulation. This, together with a very recent article which reports on Nek1 interaction with NHEJ (Non homologous end joining) repair protein Ku80, clearly establishes Nek1 as a key player in DDR signaling[29].

NEK2

Nek2 is the most studied and most well understood of the human Neks. In fact, it will be difficult to cover all of its aspects in the context of this review. Therefore, we focused on the most important features of Nek2 and would like to apologize to the many researchers whose work could not be covered here due to space restrictions.

Nek2 shares the highest sequence similarity with NIMA in its kinase domain and many biochemical, structural and functional features. This has led many researchers to believe that it may be the prototype NIMA among all vertebrate Neks and that Nek2 may maintain the primordial functions of NIMA in mitosis progression. For this reason, Nek2 became the most studied Nek family member in mammals[6]. However, care must be taken with such an interpretation since Nek2 cannot rescue NIMA defective mutants and Nek1 also shares many NIMA characteristics[30].

Nek2 expression varies during the cell cycle, being maximal between the S and G_2 phase, during which it localizes predominantly to the centrosome[31,32]. Nek2 is a component of the MTOC (microtubule organization center) at mitosis entry and a core component of the centrosome, where it phosphorylates the centrosomal key components C-Nap1 and rootletin, which form the intercentriolar linker that holds the pair of centrioles physically together. This event in turn promotes centro-

Table 1 Subcellular localization, established and possible additional functions of human and mammalian Neks

Nek	Gene/ protein synonyms	Subcellular localization	Established function	Possible additional functions (under investigation)
1	NY-REN-55 SRPS2, SRPS2A, KIAA1901	Cytoplasm, cilia, centrosome, γH2X positive DNA damage sites in nucleus	Stability and function of the primary cilium/polycystic kidney disease[14], DNA damage response to IR and chemical mutagens[2,23-25]	Meiosis[26-28], apoptosis mediated by mitochondria[13]
2	NEK2A, NLK1, RP67, HsPK21, SRPS2A	Centrosome	Regulation and promotion of centrosome segregation[33-35]	DNA damage response[127]
3	HSPK36, RP11-248G5.5	Cytoplasm	Regulation of prolactin response[41], microtubule deacetylation in neurons[47]	?
4	STK2, NRK2, pp12301	Cilia/basal bodies	Microtubule stability (silencing alters sensitivity to vincristine/taxol)[54]	DNA damage response[9], replicative senescence[9], primary cilia function[53]
5	-	?	Skeletal muscle differentiation[60], caspase-3 substrate/ apoptosis[60]	?
6	SID6-1512, RP11-101K10.6	Citotic spindle, centrosome	Mitotic spindle formation[11-12], centrosome separation[69-70]	DNA damage response[18], NF-kappa B signaling?[3,71]
7	-	Spindle poles	Mitotic spindle formation[12,88], centrosome separation[69-70]	DNA damage response?[1]
8	JCK, NEK12A, NPHP9, RHPD2	Centrosome, cilia, γH2X positive DNA damage sites in nucleus	Stability and function of the primary cilium/polycystic kidney disease[95], DNA damage response[10]	Integration of primary cilia function and DNA damage response[10]
9	NERCC, NERCC1, KIAA1995, (NEK8)	Spindle poles, centrosome, cytoplasm	Mitotic spindle formation[106], centrosome separation[100]	?
10	-	Possible centrosome/pericentriolar localization (?)	DNA damage response after UV induced damage[74]	Centrosome function?
11	-	Nucleus, nucleoli	DNA damage response induced by IR[73]	?

[1]Souza *et al*, unpublished observation.

some separation itself[33,34]. During the interphase, Nek2 is maintained in an inactive state by association with the protein kinase MST-2 and the phosphatase PP1, which keeps Nek2 dephosphorylated. After mitosis onset, polo-like kinase 1 (PLK1) phosphorylates MST-2, disrupting the trimeric complex and resulting in Nek2's activation through auto-phosphorylation. In addition, the centrosomal proteins Nlp (ninein-like protein) and centrobin contain coiled coils and are dislocated from the centrosomes in Nek2 overexpression conditions. In contrast, the Nek2 knockdown or inhibition of its catalytic activity results in the inhibition of the centrosome separation[35].

A second important functional context for Nek2 is at the spindle assembly checkpoint, where through its interaction with the major kinetochore proteins Mad1/2 and the phosphorylation of the kinetochore core protein Hec1, Nek2 may be involved in the identification of unaligned sister chromatids[36]. Failure at this checkpoint may lead to aneuploidy and other chromosomal abnormalities and knockdown or knockout of other Neks, including Nek7, has been reported to cause aneuploidy, pointing to a potential major involvement of the Nek family in the spindle assembly checkpoint[37].

Another functional context for Nek2 is in the gametogenesis, where Nek2 acts in chromatin condensation reminiscent of the role of NIMA in *Aspergillus nidulans*. In spermatocytes, the architectural chromatin protein Hmga2 is under control through phosphorylation by mitogen-activated protein kinase (MAPK) and possibly also by Nek2[38].

Finally, in *Drosophila*, Nek2 was detected at the midbody in the late mitosis and overexpression of Nek2 led to actin and actin-binding protein dislocation and cytokinesis failure, among other phenotypic effects[39].

NEK3

Nek3 is a 506 amino acid serine/threonine kinase[40] and localizes both to the nucleus and cytoplasm[41,42]. It is highly expressed in testis, prostate, ovary and brain, and shows moderate to low expression in lung and liver[40]. Its gene localizes to chromosome 13q14.2 and its mRNA is expressed in tumor, normal prostate and blood control cell lines. Insertion/deletion polymorphisms were described, in which a stretch of adenines at the end of exon 9 leads to the introduction of a premature stop codon, resulting in a truncated protein that encodes only 298 or 299 of the protein amino acids. Interestingly, this polymorphism around 13q14 is a mutational hotspot for several cancer types[43-45]. Moreover, human Nek3 has an N-terminal catalytic domain and a C-terminal regulatory domain and shares high amino acid sequence identities with mouse Nek3 (56%), but not with other NIMA-related kinases due to the absence of coiled coil regions (Figure 1)[46]. This suggests that Nek3 and its orthologs constitute a separated sub-family of the Neks[40].

Nek3 is involved in the invasion and motility of T47D cells (a human ductal breast epithelial tumor cell

line) through interaction with the guanine nucleotide exchange factor VAV2, which promotes both p21-Rac1 and transforming protein RhoA activation. These interactions are mediated by prolactin-induced association of Nek3 with the human prolactin receptor (PRLR). The signaling pathway resulting from prolactin's binding to its receptor promotes phosphorylation of paxillin, a cell adhesion mediator, and is dependent on Nek3's association with VAV2[41,42].

In its C-terminal domain, Nek3 contains a PEST motif which contains Thr475, a residue that is phosphorylated upon activation. The Thr475 and the PEST domains are phylogenetically conserved, suggesting that they are important for Nek's regulation. Expression of mutants without the Thr475 or the PEST domain cause changes in cellular morphology and polarity of both epithelial and neuronal cells. Thus, Nek3 may also be crucial to the regulation of neuronal microtubules and in disorders which involve axonal degeneration, possibly through modification of its acetylation status[47].

Another functional involvement of Nek3 with cytoskeleton components is mediated through its interaction with the EH domain-containing protein 2 (EHD2). EHD2 interacts with plasma membrane phospholipids, associates with VAV1, and forms the complex VAV1-NEK3-EHD2, which modulates p21-Rac1 activity, causing actin reorganization close to the plasma membrane at the initial stages of endocytosis[48]. In summary, Nek3 plays a role in cytoskeleton organization and dynamics through actin re-organization and may be involved in the regulation of neuronal development, endocytosis, cell motility and invasiveness of breast cancer tumor cells.

NEK4

Nek4 was initially described as serine/threonine-protein kinase 2 (STK2) by Cance *et al*[49]. In a study of a kinase specific cDNA library in human breast cancer tumors or cell lines, they identified STK2 that showed homology to *Aspergillus nidulans* NIMA and expression levels that varied widely in human breast tumors. Later, Levedakou *et al*[50] showed that STK2 is highly expressed in the heart and that its mRNA level does not vary along the cell cycle. After studies characterizing the murine STK2 the nomenclature changed to Nek4[51,52].

The human Nek4 gene is located on chromosome 3p21.1 and is transcribed into about 4kb mRNA, which encodes an 841 amino acid residue protein[50]. It is constituted by a N-terminal kinase domain and a C-terminal regulatory domain (Figure 1). Hayashi *et al*(1999)[51] described a short and a long isoform for murine Nek4. The long mNek4 isoform differs from hNek4 due to the absence of a small fragment in the regulatory domain that corresponds to an *Alu* sequence[51,52]. To date, three isoforms have been described for human Nek4. The longest canonical sequence (isoform 1: UniProtAccession P51957-1, NCBI RefSeq NM_003157) was identified by the Cance and Levedakou groups[49,50] and used to compare it to mNek4. The isoform 2 (UniProt database (UniProt Accession P51957-2 , KJ592714), is identical to mNek4 and lacks the *Alu* sequence. The isoform 3 (UniProtAccession P51957-3 and NCBI RefSeq NM_001193533) is the shortest one, with a smaller alternative N-terminal region.

Hayashi *et al*[51], (1999) showed that two isoforms of mNek4 are expressed in most tissues, except in the liver and heart where only a short isoform is expressed[50]. Recently, hNek4 expression was also observed in ciliated tissues, such as the retina, kidney tubules, brain (specifically the ventricles), heart and testis[53]. Expression in testis suggests a role in meiosis, as has been already reported for mNek4[52]. Furthermore, these new functional studies demonstrated that hNek4 depletion does not alter the cell cycle[53,54]. Therefore, as shown for other Nek family members, roles other than the regulation of the cell cycle can be attributed to Nek4, including microtubule stabilization, primary cilium assembly and, more recently, replicative senescence entry and DNA damage response[9,53,54].

Interestingly, Nek4 activity is evidenced mainly in the presence of chemotherapeutic agents. For example, in lymphoma cells, a simple Nek4 knockdown is not enough to change cell cycle or microtubule dynamics, but Nek4 knockdown triggers taxol resistance and promotes sensibility to vincristine in these cells[54]. These results indicate that Nek4 has an effect on microtubule stability in the presence of these drugs and suggests that this particularity could be explored in therapies, depending on the patient's specific levels of Nek4 protein in the tumor cells.

Besides the direct role in microtubule polymerization, Nek4 is also important for primary cilium stabilization, as was already described for Nek1 and Nek8[14,55,56]. Nek4 interacts with RPGR-interacting protein 1 (RPGRIP1) and RPGRIP1-like protein (RPGRIP1L)[53], both associated with ciliopathies. Both the eye-restricted disease "Leber Congenital Amaurosis" and the "Joubert and Meckel syndrome", which affects multiple organs, are at the severe end of the ciliopathy spectrum. After Nek4 knockdown, the number of ciliated cells decreases, but this effect is apparently not related to RPGRIP1 and RPGRIP1L phosphorylation status. This suggests that Nek4 may act as a scaffold for other cilia signaling proteins[53] and, together with Nek1 and Nek8, may be important to other ciliopathies such as PKD[14,55,56].

More recently, the role of Nek4 was also connected to the DDR because Nek4 depleted cells were found to be resistant to DNA damaging agents, such as etoposide or bleomycin, and to γ-irradiation. Besides, Nek4 interacted with DNA-PKcs, Ku70 and Ku80, proteins that have important roles in the NHEJ (non-homologous end joining) repair pathway. Nek4 depleted cells also show a decrease of histone γ-H2AX activation, probably as a result of an impairment of the DNA-PKcs recruitment[9].

NEK5

Among all members of the Nek family, Nek5 is the kinase with the least amount of information. Although identified in different organisms such as *Homo sapiens*,

Mus musculus, *Arabidopsis thaliana*, among others, there is little information about its function and localization. In humans, Nek5 is a protein of 708 amino acids, whose kinase domain is located at its N-terminus[4,8]. According to Moniz *et al*[7], Nek5 is the only member of the Nek family that has a dead box domain (Figure 1). This domain is involved in cellular processes such as pre-mRNA processing, rearrangement of ribonucleoprotein (RNP) complexes and gene expression[57]. In *Arabidopsis thaliana*, during epidermal cell expansion, Nek5 interacts with Nek4 and 6 and these interactions are important to regulate microtubule organization, probably through the phosphorylation of beta-tubulins[58]. Therefore, Nek5 may be associated with the already established cascade consisting of Nek9, 6 and 7 (see details below). However, care must be taken because the evolutionary gap between mammals and flower-plants is too large to deduce direct conclusions and the functional information on Neks in plants is even scarcer than in mammals[59]. In human cells, Nek5 is able to interact with caspase 3 and this interaction is im portant for skeletal muscle differentiation[60]. Caspase-3 is a protease involved in mechanisms such as apoptosis and cell differentiation. It was proposed by Larsen *et al*[61] that caspase-3 activates caspase-activated DNase to promote and regulate DNA strand breaks introduced into promoter regions of genes encoding effector proteins such as p21 and that this process may represent a more general mechanism of genome alterations that occur during cell differentiation. Since Nek5 interacts with caspase-3 during cell differentiation, other members of this kinase family may also be involved in differentiation associated molecular events and this possibility should be explored in future experiments.

NEK6

Unlike the other Neks, Nek6 and Nek7 are the smallest and structurally the simplest Neks, consisting only of the catalytic domain with a relatively short N-terminal extension[8]. Although they share significant similarity with each other, being about 86% identical within their catalytic domains, their N-terminal extensions are not conserved and it has been suggested that they may play a role in the differential regulation of these kinases[3,62]. SAXS experiments, together with SEC-MALS and comparative molecular modeling performed by our group revealed that hNek6 is a monomeric kinase, slightly elongated, with a flexible and disordered N-terminal domain[63].

Nek6 was initially identified in a classic biochemical screen for kinases capable of phosphorylating the hydrophobic regulatory site of the p70 ribosomal S6 kinase (S6K). Nek6 phosphorylated the Thr412 residue of S6K and other sites, *in vitro* and *in vivo*, suggesting it to be a possible regulator of this kinase[64]. Subsequently, Nek6 was described as not seeming to be responsible for the physiological phosphorylation of S6K, SGK or PKB since it was characterized as having a high preference for a Leu three residues N-terminal to the phosphorylation site of the substrate[65], and more recent evidence supports a NIMA-like mitotic role for Nek6.

Both Nek6 and Nek7 co-purify with Nek9 as a result of specific interactions and strong binding to a region located between the RCC1 domain and coiled coil motif of Nek9[66] (Figure 1). The endogenous Nek6 is activated during mitosis, concomitant with an increase in its level of expression, but this requires phosphorylation at the Ser206 residue, which is mediated through Nek9. Nek7 too is phosphorylated by Nek9 at Ser195 and both phosphorylation sites are found in the activation loops of these kinases[67]. This information led to the construction of a model in which Neks 6, 7 and 9 act as partners of the same signaling cascade[67], with Nek6/7 being substrates of Nek9. However, Nek9 remains inactive during the interphase but is activated during mitosis, phosphorylating and activating Nek6/7, which, in turn, coordinates the organization and maintenance of the mitotic spindle[66].

Overexpression of a catalytically inactive mutant of Nek6 generates cells displaying high mitotic index, defects in mitotic spindle, nuclear abnormalities and apoptosis[11]. These phenotypes are also observed from the depletion of Nek6/7 in HeLa cells using siRNA, which causes retention of cells in metaphase, with a normal chromatin condensation and alignment, but an inability to complete the segregation of chromosomes. The activity of Nek6 and also 7, therefore, seems necessary for the progression of anaphase, where the cells are either retained at the spindle assembly checkpoint (SAC), or undergo apoptosis or complete mitosis, but with an elevated risk of acquiring chromosomal abnormalities during the process[11,12]. Moreover, treatment of these depleted cells with an Aurora B inhibitor to bypass the SAC led to a reduction in the frequency of metaphase arrest, concomitant with an increase in the frequency of cells blocked in cytokinesis. Cells expressing the hypoactive mutants, even in the absence of the SAC inhibitor, also accumulated in cytokinesis. Therefore, Nek6 and Nek7 seem to have independent, non-redundant roles in mitotic spindle formation and cytokinesis: one at metaphase that requires a certain level of kinase activity and one in late mitosis that requires a higher level of activity[12].

Intriguingly, using phospho specific antibodies that detect activated Nek6, Rapley *et al*[68] showed that Nek6 activity increased 2 h after release from a nocodazole arrest, when cells would be progressing through cytokinesis. In this same study, the kinesin-related motor protein Eg5, required for spindle bipolarity, has also been described as a substrate of Nek6. It phosphorylates Eg5 kinesin *in vitro* at several residues, including Ser1033, which is also phosphorylated *in vivo* during mitosis at the spindle poles[68]. A signaling cascade seems to occur where Nek2 first phosphorylates proteins at the intercentrosomal linker in G_2 phase, resulting in their dissociation, followed by activation of Nek9 by the cyclin-dependent kinase 1 (CDK1) and the polo-like kinase 1 (PLK1) in early mitosis and subsequent activation of Nek6 and Nek7. These

kinases, in turn, phosphorylate Eg5 (previously phosphorylated by CDK1), promoting the separation of the centrosomes by the motor activity of Eg5 accumulated in the centrosomes[69,70].

Apart from roles in mitosis, human Nek6 was recently reported by our group to have a broad set of protein partners involved in diverse biological processes[3]. The hNek6 interactome showed that it is a high confidence hub kinase possibly involved in several known and novel cellular pathways, through interactions with and phosphorylation of diverse proteins. Figure 3 depicts some of the main cellular pathways identified for hNek6 based on the interacting proteins retrieved by our screenings. The novel putative pathways shown are the non-canonical Wnt signaling, Notch signaling and the actin cytoskeleton regulation, whereas the other pathways were already suggested by other studies: the nuclear factor kappa B (NF-κB) signaling[71] and the DNA damage response[18]. In regard to the DNA damage response category identified in our work, many studies show its importance among the tasks triggered by Neks[2, 8-10,18,23-25,72-74].

On the other hand, Nek6 phosphorylates the transcription factor Oct-1 (POU2F1), a potent regulator of metabolism and tumorigenicity, at S335 in the DNA binding domain during mitosis, causing Oct-1 to dissociate from the chromatin and concentrate in the centrosomes, spindle poles, kinetochores and midbody[75]. Furthermore, Nek6 phosphorylates histones H1 and H3 *in vitro*, possibly contributing to mitotic chromatin condensation[76]. Nek6 finally also binds the BTB/POZ domain-containing protein KCTD5, which appears to have a role in cytokinesis[77] and apoptosis[78].

As the other human Neks, hNek6 was recently found to be linked to carcinogenesis. It shows an increased expression and activity in gastric cancer according to the progression of the disease[79] and up-regulation of Nek6 mRNA correlates with the Peptidyl-prolyl cis-trans isomerase Pin1 up-regulation in 70% of hepatic cell carcinomas[80]. The overexpression of a catalytically inactive Nek6 promotes cell cycle arrest in human breast cancer in metaphase and leads to apoptosis[11], while its knockdown induces senescence and also apoptosis[81]. In a large-scale screening of serine/threonine kinases on different types of human tumors, Nek6 was shown to be up-regulated in non-Hodgkin's lymphoma, breast, colorectal and lung tumors[82]. Moreover, NEK6 gene, besides AURKA, has its expression increased in esophagitis and esophageal adenocarcinoma, representing a promising candidate marker of these diseases[83]. Recently, it was demonstrated that transcript, protein and kinase activity levels of Nek6 were highly elevated in malignant tumors and human cancer cell lines compared with normal tissue and fibroblast cells, indicating an important role for Nek6 in tumorigenesis[84]. Its phosphorylation at Thr210 and Ser206 is critical for the phosphorylation of STAT3 (signal transducer and activator of transcription 3) at Ser727[85]. Furthermore, its overexpression suppresses p53-induced senescence in cancer cells: it inhibits the cell cycle arrest at both G_1 and G_2/M transition, the reduction in the Cdc2 and cyclin B levels and the increase in ROS levels induced by p53[86]. Its overexpression also makes cancer cells resistant to premature senescence induced by the anti-cancer drugs camptothecin and doxorubicin[87]. The inhibition of the Nek6 function sensitizes human tumor cells to premature senescence after anti-cancer drug treatment or serum depletion[81], suggesting Nek6 to be a potential therapeutic target for various types of human cancers.

NEK7

Human Nek7 was originally described as a possible regulator of the p70 ribosomal S6 kinase[64] and of important events in the mitotic progression[12,6,67,88] (see above for Nek6). These findings have led to studies on the regulatory effects of hNek7 in key functions of the cell cycle and in cancer. The siRNA-mediated down-regulation of hNek7 and expression of kinase inactive mutants reduced centrosomal γ-tubulin levels in interphase cells and caused prometaphase arrest with defects in mitotic spindles[6,88]. Nek7 overexpression in culture cells, on the other hand, resulted in multinucleated cells and a higher proportion of apoptotic cells[89]. In the same line, the Nek7 depletion also decreased microtubule stability, while its ectopic overexpression rescued this phenotype[90]. Furthermore, hNek7 deficient mice die early in development and, on a cellular level, lack of Nek7 led to decreased chromosome numbers, increased centrosome numbers, binucleation, micronuclei formation, cytokinesis failure, growth retardation or cell death[37]. The PCM (centrosomal pericentriolar material) proteins do not accumulate at the centrosome in Nek7-depleted cells in the G_1/S and G_2/M transitions[91], indicating that Nek7 is required for centriole duplication, centrosome maturation and mitotic spindle formation[88].

The direct interaction of Nek7 with the non-catalytic domain of Nek9 allosterically activates Nek7 by interruption of its autoinhibitory conformation[92]. Consistent with these findings, recent studies demonstrated that PLK1 and CDK1 control the centrosome separation through phosphorylation and activation of Nek9 during mitosis. This leads to the Nek6/7-dependent phosphorylation of kinesin Eg5, a key event for centrosome separation and mitosis[69]. Thus, as in the case of Nek6, it is not surprising that cancer cells express elevated levels of Nek7, suggesting a role in tumor progression. Higher expression levels of Nek7 were found in larynx, breast, colorectal[82] and gall bladder cancers[93]. Taken together, these findings suggest Nek7 as a potentially important regulator of the cell cycle and reveal it as an essential component for growth and survival of mammalian cells. Furthermore, the linkage with a failure in centrosome biogenesis, chromosomal stability and ploidy, as well as the observed disturbance of microtubule dynamics connects Nek7 to hallmark features of oncogenesis.

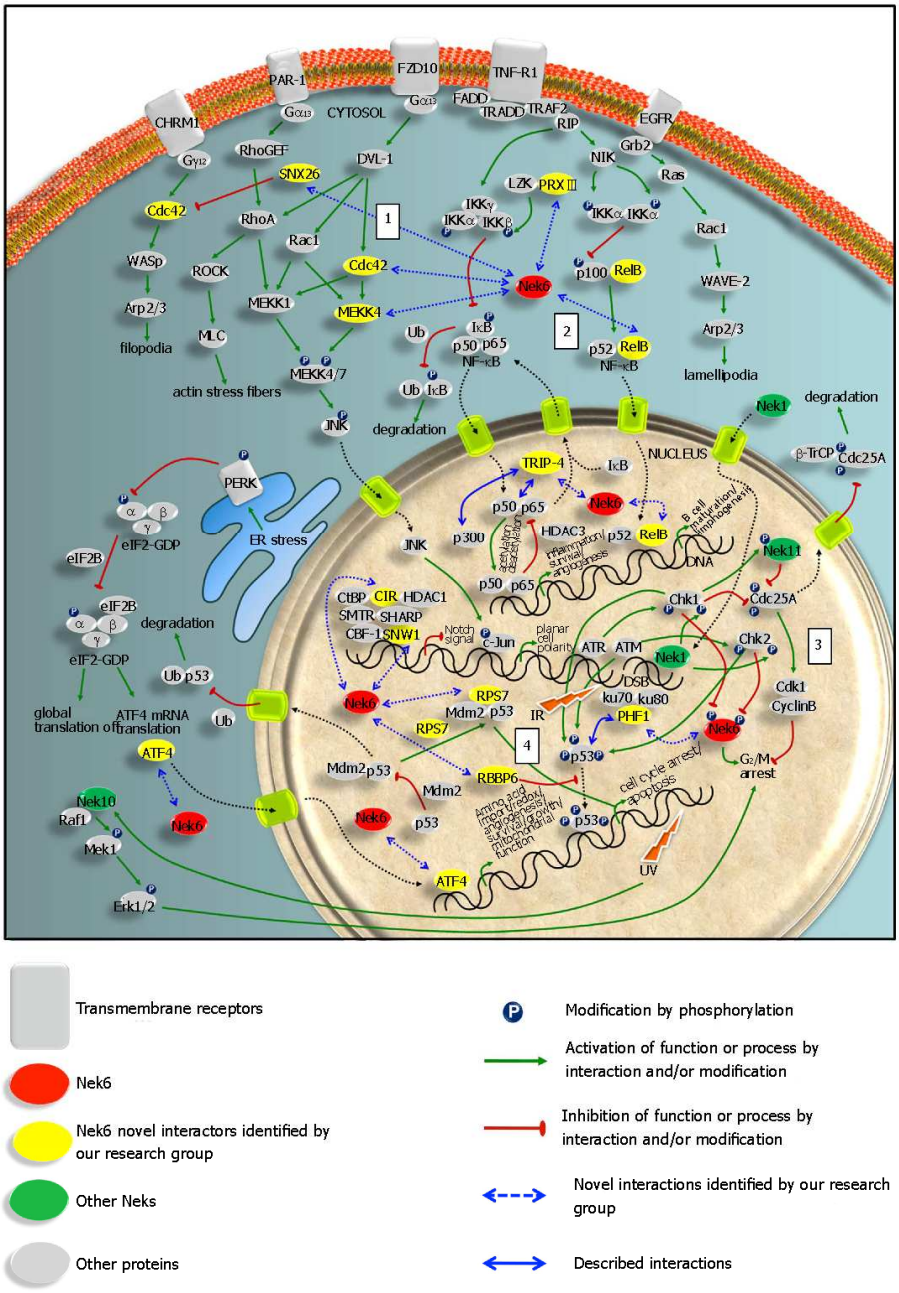

Figure 3 Nek6 interactome and the cellular functional contexts based on its interacting proteins. The four major pathways discussed in the text are: (1) actin cytoskeleton organization; (2) nuclear factor-κB signaling; (3) DNA damage response; (4) p53 signaling (according to Meirelles *et al*). See detailed legend for symbols at the bottom of the figure. IR: Ionizing radiation.

NEK8

Nek8 was first described as the mutated gene in murine autosomal recessive juvenile cystic kidney (*jck*) mice[55]. As observed for Nek1, these mutational changes found in Nek8 C-terminal domain can cause genetic kidney diseases, including polycystic kidney disease (PKD)[55]. PKD is one of the most frequent genetic kidney diseases and has a highly variable pathology, involving aberrant cell proliferation in the kidney and pleiotropic effects in multiple other organ systems, including the liver and the pancreas. Evidence that renal cyst formation is caused by defects in ciliogenesis or ciliary function is substantial[56]. In mouse cells, Nek8 localizes to the proximal region of

the primary cilium and is not observed in dividing cells[56]. In humans, Nek8 is overexpressed in primary breast tumors[94] and localizes to centrosomes and the proximal region of cilia in dividing and ciliated cells, respectively. The localization of Nek8 to centrosomes and cilia is dependent on both the kinase activity and the C-terminal non-catalytic domain homologous to RCC 1 (regulator of chromosome condensation). It is capable of autophosphorylation in the non-catalytic C-terminal region to regulate its localization or activation. Its activity is not cell cycle regulated but, in the same way as observed for Nek3, activity levels are higher in G_0-arrested cells. The kinase domain alone, although catalytically active, does not localize correctly, while a fragment containing only the RCC1 domain shows correct localization and can also be phosphorylated by Nek8[95].

Nek8 carries the causal mutations of two of the eight established mouse models of polycystic kidneys (*jck*). In these models, an abnormal interaction between Nek8 and the polycystin complex may give rise to PKD by disturbing microtubule dynamics, the mitotic spindle checkpoint and the cytoskeleton. Nek8 mutations cause overexpression of galectin-1, sorcin and vimentin and accumulation of the MUP (major urinary protein) in renal cysts of *jck* mice[96].

The role of the RCC1 domain in Nek8 is yet unknown. However, a single G448V substitution is responsible for the *jck* phenotype[55]. Overexpression of mutant forms of Nek8 (including G448V) in tissue culture cells leads to the formation of enlarged multinucleated cells and reduced numbers of actin stress fibers, although tubule cells in *jck* mice are not multinucleated, suggesting that the cellular role of Nek8 may be related to the regulation of the cytoskeleton[55].

Co-immunoprecipitation experiments demonstrated that Nek8 interacts with polycystin-2 (PKD2), a mechanosensing receptor protein, involved in the regulation of the cilium length. However, the *jck* mutation of Nek8 did not apparently affect this interaction directly. These data suggest that Nek8 interferes with the polycystic signal transduction pathways and/or the control of the targeting process of these ciliary proteins. Dysfunction of Nek8 may lead to cystogenesis by altering the structure and function of cilia in cells located at the distal nephron[97].

Recent results suggest that Nek8 has a function in the maintenance of genomic stability[10]. Loss of Nek8 leads to spontaneous DNA damage and a defect in the response of cells to replication stress. Furthermore, Nek8 interacts physically and functionally with components of the ATR-mediated DDR. The disease-related *jck* mutant of Nek8 fails to both interact with the ATR pathway proteins and to rescue the genome maintenance defects associated with Nek8 knockdown. Thus, Nek8 is a critical component of the DDR that links replication stress with primary ciliary functions and the related cystic kidney disorders[10].

NEK9

Nek9, also called Nercc1, is one of the largest Neks with 979 amino acids, with an extensive C-terminal regulatory domain, which contains seven RanGEF homology repeats, an RCC1 domain, a segment rich in Ser/Thr/Pro residues and, like in Nek2, a coiled coil dimerization motif (Figure 1)[66,98].

Nek9 was first described as Nek8 and isolated with a catalytic activity against beta-casein in rabbit lung extracts treated with IL-1, revealing the co-chromatography of a second protein homologous to the Drosophila bicaudal D protein, Bicd2, which is *in vitro* phosphorylated by Nek9 and resembles a cytoskeleton structure[99]. Moreover, Nek9 immunoprecipitation of *Xenopus laevis* egg extracts showed γ-tubulin and other members of the γ-tubulin ring complex (γ-TuRC), which are essential for the microtubule nucleating activity of the centrosome[98]. Centrosomal γ-tubulin recruitment depends on the adaptor protein NEDD1 and is controlled by PLK1. In a recent study by Sdelci *et al*[100], it was reported that PLK1 activates Nek9, which phosphorylates the Ser377 in NEDD1, promoting its recruitment together with γ-tubulin to the centrosomes of dividing cells (independently of Nek6/7). Furthermore, the microinjection of anti-Nek9 in human cells during prophase, after the chromosomes condensation, interferes in the organization of the spindles and the proper segregation of chromosomes, resulting in cell cycle arrest in prometaphase or aneuploidy[66].

Nek9 expression remains constant in different cell cycle phases (G_1/S, G_2, M, G_1); however, as observed for NIMA, there is a specific increase in its catalytic activity during mitosis, which was found to be triggered by *in vitro* and *in vivo* phosphorylation events[66]. The recombinant wild-type Nek9 shows reduced activity when extracted from exponentially growing cells, but its pre-incubation with ATP and Mg^{2+} induces its autophosphorylation at its activation loop Thr210 residue and its activation, whereas mutants lacking the coiled coil dimerization motif show significantly reduced activity[66,98]. Interestingly, the deletion of the RCC1 region leads to a catalytic hyperactivity, indicating that this region may be required for Nek9 autoinhibition[66]. Moreover, Nek9 binds to dynein light chain 1, cytoplasmic (DYNLL1), a highly conserved protein originally described as a component of the dynein complex, *via* its C-terminal (K/R) XTQT motif adjacent to Nek9 C-terminal coiled coil motif, resulting in Nek9 oligomerization, an increase in its autoactivation rate and a reduction in its binding to Nek6[101].

It is possible that Nek9 activation in mitosis involves a very small percentage (< 5%) of the total expressed protein, and in contrast with the vast majority of inactive protein, the active Nek9 (Thr210P) is first evident during prophase, concentrated at the centrosome, where it can be phosphorylated by CDK1/cyclin-B[102], until metaphase is reached. During the transition to anaphase, the immunoreactivity of Nek9 (Thr210P) decreases at the centrosomes and becomes detectable at the chromo-

somes, which is evident until telophase. Before disappearing, the active Nek9 is detected at the midbody as two points flanking the cleavage furrow during cytokinesis[98].

Due to its possible roles in the mitotic spindle organization and chromosome segregation through its activation during mitosis and interaction with Nek6/7, it is possible that most of the phenotypes observed with the microinjection of anti-Nek9 antibodies in human cells are caused by interference with Nek6/7 function[66]. Taken together, the data suggest that Nek9 is a positive upstream regulator of Nek6/7.

Among other kinases, Nek9 was recently identified by quantitative chemical proteomics as a possible marker for the diagnosis and therapy of head and neck tumors[103]. Moreover, Nek9 shows, along with other kinases implicated in cancer, its activity inhibited by the drug quercetin[104]. Its expression is increased in chronic myeloid leukemia cells resistant to imatinib[105], indicating that its up-regulation could be involved in chemotherapy resistance mechanisms. Depletion of Nek9 in glioblastoma (U1242) and renal carcinoma (Caki2) cells results in failures in cytokinesis and cell death in Caki2 cells, after overriding mitosis, and incorrect alignment of chromosomes and micronuclei formation. Therefore, it is suggested that inhibition of Nek9 is a potential anti-cancer therapeutic strategy by induction of mitotic catastrophe *via* reduced dynamics of the spindle, cytokinesis and mitotic checkpoint control[106].

NEK10

One of the most intriguing but less studied members of the Nek family is Nek10 since it has its catalytic domain flanked by two regulatory domains (Figure 1). Each of these two regulatory domains has their own peculiarities. As NIMA and Neks 1, 2, 5, 9 and 11, Nek10 also has coiled coil regions closely located to the kinase domains[8]. Furthermore, four repetitions of an armadillo repeat motif in its N-terminal regulatory domain may serve as an important region for protein-protein interactions, as has been reported for other proteins[107]. In the case of its C-terminus, a PEST region may be important to the proteolytic regulation of the protein's abundance. There are some contradictions and a debate about Nek10's full length since several different cDNAs have been deposited that differ in the C-terminal domain length.

Mutations in the Nek10 gene locus have been linked to breast cancer in different studies that were trying to find new polymorphisms in carriers of mutations in BRCA1/2 (breast cancer type 1/2 susceptibility protein)[108-110]. Moniz *et al*[74] have shown an important role for Nek10, comparing normal and tumor mammary gland cell lines. They found that Nek10 affects the ERK1/2 (extracellular signal-regulated kinase 1/2) signaling pathway, after activation with UV radiation. Nek10 has been shown to form a functional complex with RAF1 and MEK1 (dual specificity mitogen-activated protein kinase kinase 1). In this sense, cell cycle arrest in G_2/M was observed and Nek10 caused both MEK1 activation and the ERK1/2 phosphorylation. However, these preliminary data suggest a possible involvement of Nek10 in the DDR, as already demonstrated for Nek1, 4, 6, 8 and 11[2,8-10,18,23-25,72-73]. Moreover, like BRCA1 and BRCA2, Nek10 may be a therapeutic target in breast cancer.

NEK11

Nek11 is one of the least studied Nek family members and has the highest sequence similarity to Nek4. Its gene is present on the same chromosome as that of Nek4 but on the long arm (3q22-1). Nek11 was first identified by Noguchi *et al* (2002)[111] and shows a high sequence similarity with Nek4 and 3 in its kinase domain, but is more similar to Nek2 in its regulatory region (Figure 1). Interestingly, Noguchi *et al*[111] have not found Nek4/11-related kinases in *C. elegans* or *D. melanogaster*, suggesting that the Nek11-containing subfamily may have only appeared through gene or genome duplication after separation of the deuterostome branch in the animal kingdom[111].

Noguchi *et al*[111] (2002) described two isoforms for Nek11. The longer isoform (Nek11L) is composed of 645 residues, while the shorter one (Nek11S) contains only 470 residues. Nek11 shows a N-terminal kinase domain and a C-terminal regulatory domain with a coiled coil and three PEST sequences, suggesting a proteolytic, cell cycle specific regulation of its expression. Nek11, different from Nek1, 2 and 4, is not present in a higher quantity in the testis or ovary, but its mRNA is found in the brain's cerebellum, trachea, lung, appendix and uterus[111]. Another important difference to Nek4 is that Nek11 shows a timely cell cycle related expression pattern, relating it closer to Nek2, with both showing an expression peak at the G_2/M transition.

The first indication that Nek11 could be important in the regulation of cell cycle checkpoints was the identification of histones H1, H2A and H3 as Nek11 phosphorylation substrates. Furthermore, in the presence of genotoxic agents, Nek11 showed both an increased expression and activity at the G_2/M transition. Although this is decreased by caffeine, suggesting that Nek11 DDR may be associated with the ATM/ATR pathways, which also showed the same inhibition by caffeine[111].

Another common point between Nek11 and Nek2 is their localization to the nucleolus. In the study of Noguchi *et al*[112] (2004), it was observed that in U2OS cells Nek11L is present in the nucleolus during interphase and telophase and that it probably interacts with Nek2A in the nucleolus. Moreover, Noguchi *et al*[112]speculated that Nek2A could phosphorylate Nek11L C-terminal and, in this way, antagonize its auto-inhibitory function, which would cause Nek11 activation in G_1/S arrested cells[112].

Recently, some of Noguchi's results were followed up by Melixetian *et al*[73]. This study points to Nek11 as an important player in cancer development. Melixetian *et al*[73] observed that Nek11 depleted U2OS cells lose an important G_2/M checkpoint after IR. In this way, it was

verified that after IR Chk1 phosphorylates both M-phase inducer phosphatase 1 (CDC25A) and Nek11. Nek11 in turn also phosphorylates CDC25A, leading to its proteasomal degradation and subsequent inhibition of cyclins followed by a cell cycle arrest at the G_2/M transition.

The studies involving Nek11 so far point to it as an important protein for the cell cycle regulation in the context of the DDR. However, more interactome studies are required to clarify other possible functions of Nek11 in the cell.

DISCUSSION

After knowing sufficient details on all of the eleven individual Neks, we will now return to a more general and integrative approach and try to find common functional contexts for the family as a whole in human cells. As pointed out in the introduction, Neks may be assigned to three major functional contexts: (1) centrioles and mitotic spindle functions; (2) primary ciliary function; and (3) G_2/M phase associated DDR. Although most individual Neks have been associated with one main context, recent functional data as well as the identification of interaction partners for several Neks from two or even all three contexts may suggest that Neks have a broader function, possibly on a regulatory level, that consequently affects the three main functions. A first way of looking at this is by comparing the interaction profiles and functional contexts of the published interacting partners, summarized in Figure 2, which shows the Neks global interaction profile and the possible new biological processes in which they are involved due to their interaction with multiple proteins.

Several protein interactors with violet color interact with Nek1, 2, 3, 8, 9 and 11 and can be described as associated with the "axon guidance"/transport processes. They include, for example, fasciculation and elongation protein zeta (FEZ)-1 and 2 that interact with Nek1[2,113,114].

Several proteins associated with apoptotic processes interact with Nek6: serine/threonine-protein kinase PAK 6 (PAK6), serine/threonine-protein kinase Sgk1 (SGK1) and DBIRD complex subunit KIAA1967 (KIAA1967) (darker green color).

Nek9 interacts with several proteins from the autophagy-related protein 8 family (GABARAP, GABARAPL1, GABARAPL2, MAP1LC3A, MAP1LC3B and MAP1LC3C) (light blue).

Several proteins from DNA repair processes interact with either Nek1, 6, 9 or 10: RuvB-like 2 (RUVBL2), Fanconi anemia group I protein (FANCI), transcriptional regulator ATRX (ATRX), FACT complex subunit SSRP1 (SSRP1) and SUMO-1 (SUMO1) (red). The putative DNA repair and recombination protein RAD26-like (RAD26L), the PHD finger protein 1 (PHF1), and also the double-strand-break repair protein rad21 homolog (RAD21, not shown in Figure 2), all identified as Nek6 interactors in our yeast two-hybrid screens[3], are also possibly involved in the DDR[115,116].

In order to demonstrate the potential discovery of additional functional contexts through interactomics studies, we will now have a closer look at the Nek6 interactome as described by our group[3] (Figure 3). Novel Nek6 interacting partners are indicated by yellow ellipses and suggest the following new functional contexts: (1) Nek6 is possibly involved in actin cytoskeleton organization through its interaction with cell division control protein 42 homolog (CDC42) and sorting nexin-26 (SNX26)[3]. Since SNX26 has a negative regulatory role on CDC42 and Nek6 interacts with both of them, the final output of Nek6 must be addressed by future experiments. However, these findings are supported by the fact that for Nek3 a clear involvement in related processes has been reported (see Nek3 section above); (2) Nek6 may be involved in the activation of the NF-κB signaling on multiple layers, since it interacts with the transcription factor RelB, Prx-Ⅲ and/or TRIP-4[3,71]. Matsuda *et al*[71] found Nek6 as an activating protein in a siRNA knockdown screen to identify proteins that participate in the regulation of cellular survival transcription factor NF-κB[71]. The regulation may occur on several levels: through direct phosphorylation, interaction or regulation of the nuclear translocation of key components of the NF-κB complex, like RelB, or even on the transcriptional level. The latter seems likely, since Nek6 also interacts with SNW domain-containing protein 1 (SNW1) and a PHF domain containing protein (PHF1)[3], both of which have been recently identified as key components involved in the complex, multiprotein machinery involved in the transcriptional activation of the NF-κB gene[117]. Again, Nek6 regulatory role here may be mediated through interaction and/or phosphorylation; (3) the IR-induced DNA damage response is mediated by Nek1, 6 and 11, leading to cell cycle arrest[18,23,25,72,73]. The UV-induced DNA damage response is mediated by Nek10, also leading to cell cycle arrest[74]. This may suggest that different Neks may have specialized to mediate different forms of DNA damage responses; and (4) it is known that Nek6 can counteract p53 induced senescence[86]. As we can observe in Figure 3, this may occur indirectly through Nek6 modulation of p53 interactors 40S ribosomal protein S7 (RPS7) and/or E3 ubiquitin-protein ligase RBBP6 (RBBP6). It is worth noting here that Nek4 has the opposite effect of Nek6. Nek4 seems to be required for the cell to enter in senescence[9].

Another important point is the finding that certain functions first only described for isolated specific Neks have been later confirmed for most if not all other Neks. Nek1 was the first family member to be associated with DDR signaling events[23]. In our yeast two-hybrid screen to identify Nek1 interacting proteins, we identified proteins involved in the repair process itself (MRE11A) and in different signaling pathways associated with it (ATRX, PPP2R5 A/D, YWHAH, TP53BP1) (Figure 4).

Nek4, 6, 8, 10 and 11 have also been reported to physically interact with key members of DDR pathways or to interfere functionally in signaling cascades in a

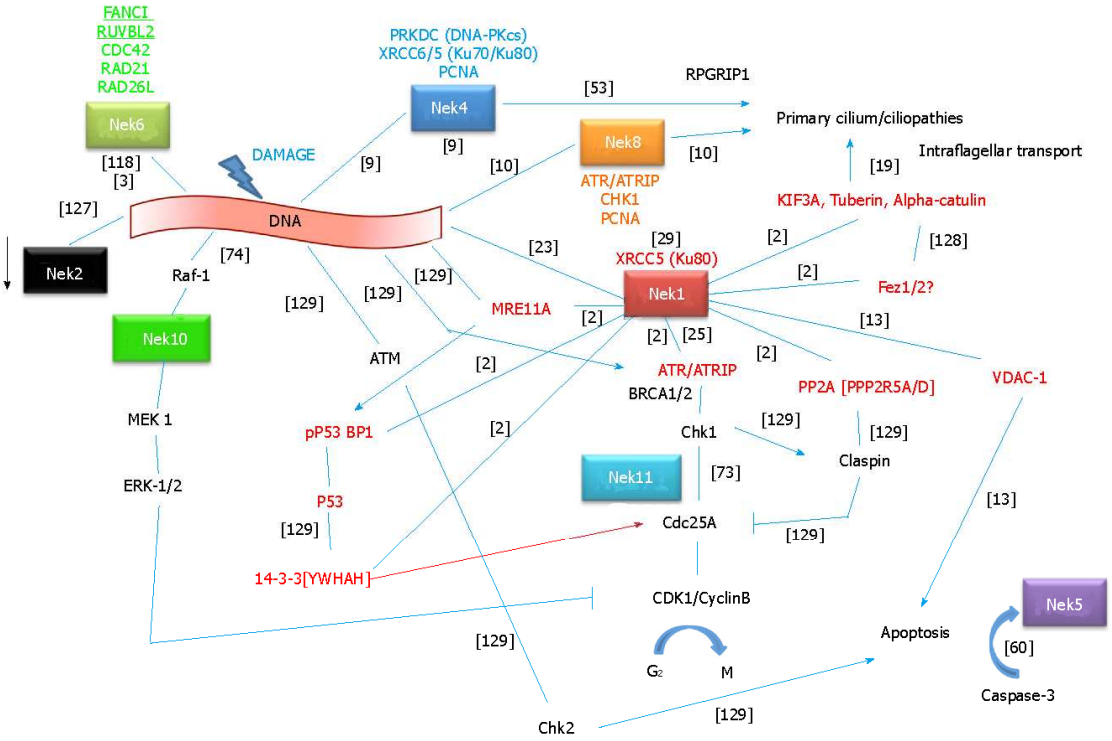

Figure 4 Nek1 interactome and crosstalk with other Neks and protein interactors in the context of the DNA damage response pathways. Interactions between proteins are depicted as simple lines, activation is depicted as an arrow and inhibition as an arrow with a line as arrowhead. A red arrow for 14-3-3 means that it causes activation by the transport of CDC25 to the nucleus. Nek1 interacted with a specific 14-3-3 isoform called YWHAH[2] (gene symbols inside brackets correspond to the isoforms of those proteins which were described to interact with Nek1). Not necessarily the same specific 14-3-3 protein promotes the indicated functions. Rather, a family characteristic is intended to be assigned. Nek2 kinase activity is inhibited after DNA damage (arrow)[127]. The red protein names are those that have been identified to directly interact with Nek1 as identified by the yeast two-hybrid system[2] or other as indicated in the figure. Gene symbols above/under protein names represent other interactors of those proteins. Nek4 interactors have been identified by mass spectrometry[9]. As can be seen, all but three Neks (Nek3, 7 and 9) seem to be directly linked to the DNA damage response. Most strikingly, we can see a direct connection for Nek8, 4 and 1 between DDR and primary cilium function and ciliopathies. New connections to apoptosis have been recently pointed out for Nek1 and 5. References for interactions are depicted in brackets: Nek6[3,118]; Nek1[2,13,25]; Nek4[9,53]; Nek8[10]; Nek11[73]; Nek10[74]; Nek2[127]; Nek5[60]; KIF3A[19]; Fez1/2[128], various known interactions[129].

broader context of the G2/M transition[8-10,18,73-74]. As described above for Nek6, the interactors RAD26L, PHF1, RAD21[3], FANCI and RUVBL2[118] are all associated with the DDR. Together with the relatively recent work by Lee *et al* (2008)[18], this suggests Nek6 may also interfere in DDR. However, the stimuli that activate such possible pathways *via* Nek6 are still unknown. In further yeast two-hybrid screens and mass spectrometry interactomics studies we found other DDR members interacting with Nek3, 4, 5, 7, 8, and 10 (unpublished data). Recent publications clearly confirmed part of those findings or went beyond them by characterizing this new involvement not only functionally, but also establishing possible cross-connections between primary cilia signaling and DDR in the case of Nek8[10]. For Nek4, an involvement in senescence signaling was established and in mass spectrometry experiments, several DDR proteins such as DNA-PKcs (PRKDC), Ku70/Ku80 (XRCC6/5) and PCNA were identified as Nek4 interacting proteins (Figure 4)[9]. Furthermore, Nek4 has been reported to interact with RPGRIP at the primary cilium[53], thereby establishing another link between DDR and primary cilium function.

A new role for Nek5 in differentiation and apoptosis signaling has been identified and characterized through its interaction with and proteolytic processing by caspase-3[60]. Evidently, apoptosis signaling is closely related to DDR and the G2/M checkpoint because cells unable to repair major DNA damage must either halt in the cycle or be dispatched by apoptosis. The link between Neks, DDR and apoptosis is not new as Chen *et al*[13] had also already reported an interaction of Nek1 with mitochondrial VDAC1. Nek1 phosphorylates VDAC1 and prevents apoptosis by avoiding VDAC1 opening and leakage of cytochrome c, which would activate apoptotic caspases. The down-regulation of Nek1 protein level or kinase activity through apoptosis signaling decreases VDAC1 phosphorylation and results in its opening and leakage of cytochrome c, thereby activating the apoptosis program.

For Nek1, the coexistence of functional roles in both DDR and ciliopathies and primary cilia function has been long established (Figure 4). Nek1 interacts with several proteins involved in the primary cilia function

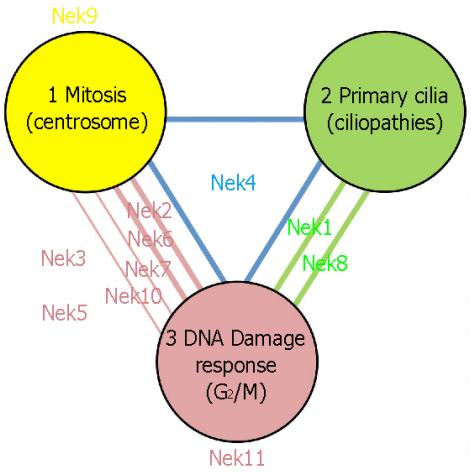

Figure 5 Functional overlap in the human Nek kinase family: seven of eleven Neks participate in two and one Nek in all three of the main core functions of the Nek family (centrosome-related mitosis, primary cilia and DNA damage response). The three corners of the triangle represent each a key concept function for the Nek family, *e.g.*, Nek9 and 11 sole involvement in mitosis[66,67] and DDR[73] respectively, has been well documented. The Nek names and bold lines represent cases where accumulated experimental evidence strongly suggests a regulatory role for that Nek in that context or in both of the contexts the line connects: Nek1[2,22,23]; Nek2[123]; Nek4[8,53] (Basei *et al* unpublished); Nek6[8]; Nek7[67]; Nek8[8,10]; Nek10[74]. The thinner lines represent our own group's preliminary or unpublished interaction data (both from yeast two-hybrid system and immunoprecipitation coupled to mass spectrometry analysis data), suggestive of a participation of that Nek in both connected functions (Nek7: Souza *et al*, unpublished).

and especially in kidney duct mechanosensing (KIF3A, tuberin, alpha-catulin, polycystin 1/2). Mutations in the genes that encode all of these proteins like those that cause expression of truncated non-functional Nek1 itself, cause PKD[14]. Since Nek8 is functionally and evolutionary most closely related to Nek1 among the Nek family, it came as no surprise that Nek8 mutations were also found to cause ciliopathies and cystic kidney disease. Moreover, Nek8 interacts with some key DDR proteins, including ATR, Chk1 and PCNA, just like Nek1[10]. What is new in these milestone discoveries, however, is the possibility that somehow these two pathways are causative or coincidentally connected. Choi *et al*[10] made the observation that mice cells with diminished Nek8 kinase activity, simulating a kidney ciliopathy, already show a constitutive activation of DDR pathways in the embryonic phase, as evidenced by repair foci in their kidney cells nuclei. This raises a couple of possibilities to consider: either the cilia have some function in the sensing of DNA damage or in transmitting downstream events, or otherwise, the cilia defects somehow transduce (*via* Nek8) to a possible lack of repair of replication defects. Of course a simpler explanation could be that both phenomena are affected simply because Nek8 participates in both of them simultaneously. However, an additional possibility is that Nek8 acts on a higher regulatory level that coordinates both pathways based on the necessity of the cell to coordinate these events closely during the course of the cell cycle. Clearly, further studies are necessary to evaluate these new possibilities. However, it seems to be clear now that the three central functions controlled by Neks, mitosis, primary cilia and DDR, are more connected than previously expected and that several if not all Neks participate in more than one of them.

A possibility exists that the Neks *per se* are the key regulatory elements that may connect these three functions. The seemingly functional redundancy may in fact rather represent connecting elements between hitherto non-connected regulatory circuits (Figure 5), *e.g.*, between primary ciliary function and DDR for Nek8[10] and Nek1[2,23,14]. Furthermore, these circuits may cooperate in a concerted one or two-directional fashion (Nek8).

Most interestingly, from a cilium perspective, recent evidence also indicates a strong link between cilia, stress responses and DNA damage repair processes. A recent study showed that environmental stresses, including UV and IR, result in altering the protein composition of centriolar satellites, thereby promoting de novo ciliogenesis[119]. Together with the recent findings that ciliopathy-associated mutations in DNA damage key regulators (*e.g.*, Mre, Znf423) also connect cilia and DDR[120-124], it is tempting to speculate that cilia may act as platforms for cell cycle checkpoints or the DDR.

CONCLUSION

Clearly, the past 10 years have provided new and exciting insights into the multifaceted functions of this interesting protein kinase family and the future promises to hold more surprises and the discovery of new functional connections. An exciting time has come to the field of Nek research and the Neks are ready to step out of the shade and take a main role along the other important cell cycle regulatory kinases: Polo-like kinases, Aurora kinases and Cyclin-dependent kinases. It is time to stop Ne(c)king around with them and allow them to enter the spot light in the field of cell cycle biology.

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REFERENCES

1 **De Souza CP**, Horn KP, Masker K, Osmani AS. The SNOB (NUP98) nucleoporin interact with the NIMA kinase in Aspergillusnidulans. *Genetics* 2003; **165:** 1071-1081 [PMID: 14668365]

2 **Surpili MJ**, Delben TM, Kobarg J. Identification of proteins that interact with the central coiled-coil region of the human protein kinase NEK1. *Biochemistry* 2003; **42:** 15369-15376 [PMID: 14690447 DOI: 10.1021/bi034575v]

3 **Vaz Meirelles G**, Ferreira Lanza DC, da Silva JC, Santana Bernachi J, Paes Leme AF, Kobarg J. Characterization of hNek6 interactome reveals an important role for its short N-terminal domain and colocalization with proteins at the centrosome. *J Proteome Res* 2010; **9:** 6298-6316 [PMID: 20873783 DOI: 10.1021/pr100562w]

4 **O'Connell MJ**, Krien MJ, Hunter T. Never say never. The NIMA-related protein kinases in mitotic control. *Trends Cell Biol* 2003; **13**: 221-228 [PMID: 12742165 DOI: 10.1016/S0962-8924(03)00056-4]

5 **Quarmby LM**, Mahjoub MR. Caught Nek-ing: cilia and centrioles. *J Cell Sci* 2005; **118**: 5161-5169 [PMID: 16280549 DOI: 10.1242/jcs.02681]

6 **O'regan L**, Blot J, Fry AM. Mitotic regulation by NIMA-related kinases. *Cell Div* 2007; **2**: 25 [PMID: 17727698 DOI: 10.1186/1747-1028-2-25]

7 **Moniz L**, Dutt P, Haider N, Stambolic V. Nek family of kinases in cell cycle, checkpoint control and cancer. *Cell Div* 2011; **6**: 18 [PMID: 22040655 DOI: 10.1186/1747-1028-6-18]

8 **Fry AM**, O'Regan L, Sabir SR, Bayliss R. Cell cycle regulation by the NEK family of protein kinases. *J Cell Sci* 2012; **125**: 4423-4433 [PMID: 23132929 DOI: 10.1242/jcs.111195]

9 **Nguyen CL**, Possemato R, Bauerlein EL, Xie A, Scully R, Hahn WC. Nek4 regulates entry into replicative senescence and the response to DNA damage in human fibroblasts. *Mol Cell Biol* 2012; **32**: 3963-3977 [PMID: 22851694 DOI: 10.1128/MCB.00436-12]

10 **Choi HJC**, Lin JR, Vannier JB, Slaats GG, Kile AC, Paulsen RD. NEK8 Links the ATR-Regulated Replication Stress Response and S Phase CDK Activity to Renal Ciliopathies. *Molecular Cell* 2013; **51**: 423-439 [DOI: 10.1016/j.molcel.2013.08.006]

11 **Yin MJ**, Shao L, Voehringer D, Smeal T, Jallal B. The serine/threonine kinase Nek6 is required for cell cycle progression through mitosis. *J Biol Chem* 2003; **278**: 52454-52460 [PMID: 14563848 DOI: 10.1074/jbc.M308080200]

12 **O'Regan L**, Fry AM. The Nek6 and Nek7 protein kinases are required for robust mitotic spindle formation and cytokinesis. *Mol Cell Biol* 2009; **29**: 3975-3990 [PMID: 19414596 DOI: 10.1128/MCB.01867-08]

13 **Chen Y**, Craigen WJ, Riley DJ. Nek1 regulates cell death and mitochondrial membrane permeability through phosphorylation of VDAC1. *Cell Cycle* 2009; **8**: 257-267 [PMID: 19158487 DOI: 104161/cc.8.2.7551]

14 **Upadhya P**, Birkenmeier EH, Birkenmeier CS, Barker JE. Mutations in a NIMA-related kinase gene, Nek1, cause pleiotropic effects including a progressive polycystic kidney disease in mice. *Proc Natl Acad Sci USA* 2000; **97**: 217-221 [PMID: 10618398 DOI: 10.1073/pnas.97.1.217]

15 **Wilson PD**. Polycystic kidney disease: new understanding in the pathogenesis. *Int J Biochem Cell Biol* 2004; **36**: 1868-1873 [PMID: 15203099]

16 **Thiel C**, Kessler K, Giessl A, Dimmler A, Shalev SA, von der Haar S, Zenker M, Zahnleiter D, Stöss H, Beinder E, Abou Jamra R, Ekici AB, Schröder-Kress N, Aigner T, Kirchner T, Reis A, Brandstätter JH, Rauch A. NEK1 mutations cause short-rib polydactyly syndrome type majewski. *Am J Hum Genet* 2011; **88**: 106-114 [PMID: 21211617]

17 **Chen CP**, Chang TY, Tzen CY, Wang W. Second-trimester sonographic detection of short rib-polydactyly syndrome type II (Majewski) following an abnormal maternal serum biochemical screening result. *Prenat Diagn* 2003; **23**: 353-355 [PMID: 12673646 DOI: 10.1002/pd.574]

18 **Lee MY**, Kim HJ, Kim MA, Jee HJ, Kim AJ, Bae YS, Park JI, Chung JH, Yun J. Nek6 is involved in G2/M phase cell cycle arrest through DNA damage-induced phosphorylation. *Cell Cycle* 2008; **7**: 2705-2709 [PMID: 18728393 DOI: 10.4161/cc.7.17.6551]

19 **Lin F**, Hiesberger T, Cordes K, Sinclair AM, Goldstein LS, Somlo S, Igarashi P. Kidney-specific inactivation of the KIF3A subunit of kinesin-II inhibits renal ciliogenesis and produces polycystic kidney disease. *Proc Natl Acad Sci USA* 2003; **100**: 5286-5291 [PMID: 12672950 DOI: 10.1073/pnas.0836980100]

20 **Kleymenova E**, Ibraghimov-Beskrovnaya O, Kugoh H, Everitt J, Xu H, Kiguchi K, Landes G, Harris P, Walker C. Tuberin-dependent membrane localization of polycystin-1: a functional link between polycystic kidney disease and the TSC2 tumor suppressor gene. *Mol Cell* 2001; **7**: 823-832 [PMID: 11336705 DOI: 10.1016/S1097-2765(01)00226-X]

21 **Huan Y**, van Adelsberg J. Polycystin-1, the PKD1 gene product, is in a complex containing E-cadherin and the catenins. *J Clin Invest* 1999; **104**: 1459-1468 [PMID: 10562308 DOI: 10.1172/JCI5111]

22 **Mahjoub MR**, Qasim Rasi M, Quarmby LM. A NIMA-related kinase, Fa2p, localizes to a novel site in the proximal cilia of Chlamydomonas and mouse kidney cells. *Mol Biol Cell* 2004; **15**: 5172-5186 [PMID: 15371535 DOI: 10.1091/mbc.E04-07-0571]

23 **Polci R**, Peng A, Chen PL, Riley DJ, Chen Y. NIMA-related protein kinase 1 is involved early in the ionizing radiation-induced DNA damage response. *Cancer Res* 2004; **64**: 8800-8803 [PMID: 15604234 DOI: 10.1158/0008-5472.CAN-04-2243]

24 **Pelegrini AL**, Moura DJ, Brenner BL, Ledur PF, Maques GP, Henriques JA, Saffi J, Lenz G. Nek1 silencing slows down DNA repair and blocks DNA damage-induced cell cycle arrest. *Mutagenesis* 2010; **25**: 447-454 [PMID: 20501547 DOI: 10.1093/mutage/geq026]

25 **Liu S**, Ho CK, Ouyang J, Zou L. Nek1 kinase associates with ATR-ATRIP and primes ATR for efficient DNA damage signaling. *Proc Natl Acad Sci USA* 2013; **110**: 2175-2180 [PMID: 23345434 DOI: 10.1073/pnas.1217781110]

26 **Letwin K**, Mizzen L, Motro B, Ben-David Y, Bernstein A, Pawson T. A mammalian dual specificity protein kinase, Nek1, is related to the NIMA cell cycle regulator and highly expressed in meiotic germ cells. *EMBO J* 1992; **11**: 3521-3531 [PMID: 1382974]

27 **Feige E**, Chen A, Motro B. Nurit, a novel leucine-zipper protein, expressed uniquely in the spermatid flower-like structure. *Mech Dev* 2002; **117**: 369-377 [PMID: 12204287 DOI: 10.1016/S0925-4773(02)00217-4]

28 **Holloway K**, Roberson EC, Corbett KL, Kolas NK, Nieves E, Cohen PE. NEK1 Facilitates Cohesin Removal during Mammalian Spermatogenesis. *Genes* (Basel) 2011; **2**: 260-279[PMID: 21931878]

29 **Patil M**, Pabla N, Ding HF, Dong Z. Nek1 interacts with Ku80 to assist chromatin loading of replication factors and S-phase progression. *Cell Cycle* 2013; **12**: 2608-2616 [PMID: 23851348 DOI: 10.4161/cc.25624]

30 **Feige E**, Shalom O, Tsuriel S, Yissachar N, Motro B. Nek1 shares structural and functional similarities with NIMA kinase. *Biochim Biophys Acta* 2006; **1763**: 272-281 [PMID: 16603261 DOI: 10.1016/j.bbamcr.2006.01.009]

31 **Fry AM**, Schultz SJ, Bartek J, Nigg EA. Substrate specificity and cell cycle regulation of the Nek2 protein kinase, a potential human homolog of the mitotic regulator NIMA of Aspergillus nidulans. *J Biol Chem* 1995; **270**: 12899-12905 [PMID: 7759549 DOI: 10.1074/jbc.270.21.12899]

32 **Fry AM**, Arnaud L, Nigg EA. Activity of the human centrosomal kinase, Nek2, depends on an unusual leucine zipper dimerization motif. *J Biol Chem* 1999; **274**: 16304-16310 [PMID: 10347187 DOI: 10.1074/jbc.274.23.16304]

33 **Fry AM**, Mayor T, Meraldi P, Stierhof YD, Tanaka K, Nigg EA. C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle-regulated protein kinase Nek2. *J Cell Biol* 1998; **141**: 1563-1574 [PMID: 9647649 DOI: 10.1083/jcb.141.7.1563]

34 **Yang J**, Adamian M, Li T. Rootletin interacts with C-Nap1 and may function as a physical linker between the pair of centrioles/basal bodies in cells. *Mol Biol Cell* 2006; **17**: 1033-1040 [PMID: 16339073 DOI: 10.1091/mbc.E05-10-0943]

35 **Rellos P**, Ivins FJ, Baxter JE, Pike A, Nott TJ, Parkinson DM, Das S, Howell S, Fedorov O, Shen QY, Fry AM, Knapp S,

Smerdon SJ. Structure and regulation of the human Nek2 centrosomal kinase. *J Biol Chem* 2007; **282:** 6833-6842 [PMID: 17197699 DOI: 10.1074/jbc.M609721200]
36 **Wei R**, Ngo B, Wu G, Lee WH. Phosphorylation of the Ndc80 complex protein, HEC1, by Nek2 kinase modulates chromosome alignment and signaling of the spindle assembly checkpoint. *Mol Biol Cell* 2011; **22:** 3584-3594 [PMID: 21832156 DOI: 10.1091/mbc.E11-01-0012]
37 **Salem H**, Rachmin I, Yissachar N, Cohen S, Amiel A, Haffner R, Lavi L, Motro B. Nek7 kinase targeting leads to early mortality, cytokinesis disturbance and polyploidy. *Oncogene* 2010; **29:** 4046-4057 [PMID: 20473324 DOI: 10.1038/onc.2010]
38 **Di Agostino S**, Fedele M, Chieffi P, Fusco A, Rossi P, Geremia R, Sette C. Phosphorylation of high-mobility group protein A2 by Nek2 kinase during the first meiotic division in mouse spermatocytes. *Mol Biol Cell* 2004; **15:** 1224-1232 [PMID: 14668482 DOI: 10.1091/mbc.E03-09-0638]
39 **Prigent C**, Glover DM, Giet R. Drosophila Nek2 protein kinase knockdown leads to centrosome maturation defects while overexpression causes centrosome fragmentation and cytokinesis failure. *Exp Cell Res* 2005; **303:** 1-13 [PMID: 15572022 DOI: 10.1016/j.yexcr.2004.04.052]
40 **Kimura M**, Okano Y. Molecular cloning and characterization of the human NIMA-related protein kinase 3 gene (NEK3). *Cytogenet Cell Genet* 2001; **95:** 177-182 [PMID: 12063396 DOI: 10.1074/jbc.274.19.13491]
41 **Miller SL**, DeMaria JE, Freier DO, Riegel AM, Clevenger CV. Novel association of Vav2 and Nek3 modulates signaling through the human prolactin receptor. *Mol Endocrinol* 2005; **19:** 939-949 [PMID: 15618286 DOI: 10.1210/me.2004-0443]
42 **Miller SL**, Antico G, Raghunath PN, Tomaszewski JE, Clevenger CV. Nek3 kinase regulates prolactin-mediated cytoskeletal reorganization and motility of breast cancer cells. *Oncogene* 2007; **26:** 4668-4678 [PMID: 17297458 DOI: 10.1038/sj.onc.1210264]
43 **Hernández M**, Almeida TA. Is there any association between nek3 and cancers with frequent 13q14 deletion? *Cancer Invest* 2006; **24:** 682-688 [PMID: 17118778 DOI: 10.1080/07357900600981364]
44 **Kytölä S**, Farnebo F, Obara T, Isola J, Grimelius L, Farnebo LO, Sandelin K, Larsson C. Patterns of chromosomal imbalances in parathyroid carcinomas. *Am J Pathol* 2000; **157:** 579-586 [PMID: 10934160 DOI: 10.1016/S0002-9440(10)64568-3]
45 **Shaughnessy J**, Tian E, Sawyer J, Bumm K, Landes R, Badros A, Morris C, Tricot G, Epstein J, Barlogie B. High incidence of chromosome 13 deletion in multiple myeloma detected by multiprobe interphase FISH. *Blood* 2000; **96:** 1505-1511 [PMID: 10942398]
46 **Schultz SJ**, Fry AM, Sütterlin C, Ried T, Nigg EA. Cell cycle-dependent expression of Nek2, a novel human protein kinase related to the NIMA mitotic regulator of Aspergillus nidulans. *Cell Growth Differ* 1994; **5:** 625-635 [PMID: 7522034]
47 **Chang J**, Baloh RH, Milbrandt J. The NIMA-family kinase Nek3 regulates microtubule acetylation in neurons. *J Cell Sci* 2009; **122:** 2274-2282 [PMID: 19509051 DOI: 10.1242/jcs.048975]
48 **Benjamin S**, Weidberg H, Rapaport D, Pekar O, Nudelman M, Segal D, Hirschberg K, Katzav S, Ehrlich M, Horowitz M. EHD2 mediates trafficking from the plasma membrane by modulating Rac1 activity. *Biochem J* 2011; **439:** 433-442 [PMID: 21756249 DOI: 10.1042/BJ20111010]
49 **Cance WG**, Craven RJ, Weiner TM, Liu ET. Novel protein kinases expressed in human breast cancer. *Int J Cancer* 1993; **54:** 571-577 [PMID: 8099900 DOI: 10.1002/ijc.2910540409]
50 **Levedakou EN**, He M, Baptist EW, Craven RJ, Cance WG, Welcsh PL, Simmons A, Naylor SL, Leach RJ, Lewis TB. Two novel human serine/threonine kinases with homologies to the cell cycle regulating Xenopus MO15, and NIMA kinases: cloning and characterization of their expression pattern. *Oncogene* 1994; **9:** 1977-1988 [PMID: 8208544]
51 **Hayashi K**, Igarashi H, Ogawa M, Sakaguchi N. Activity and substrate specificity of the murine STK2 Serine/Threonine kinase that is structurally related to the mitotic regulator protein NIMA of Aspergillus nidulans. *Biochem Biophys Res Commun* 1999; **264:** 449-456 [PMID: 10529384 DOI: 10.1006/bbrc.1999.1536]
52 **Chen A**, Yanai A, Arama E, Kilfin G, Motro B. NIMA-related kinases: isolation and characterization of murine nek3 and nek4 cDNAs, and chromosomal localization of nek1, nek2 and nek3. *Gene* 1999; **234:** 127-137 [PMID: 10393247 DOI: 10.1016/S0378-1119(99)00165-1]
53 **Coene KL**, Mans DA, Boldt K, Gloeckner CJ, van Reeuwijk J, Bolat E, Roosing S, Letteboer SJ, Peters TA, Cremers FP, Ueffing M, Roepman R. The ciliopathy-associated protein homologs RPGRIP1 and RPGRIP1L are linked to cilium integrity through interaction with Nek4 serine/threonine kinase. *Hum Mol Genet* 2011; **20:** 3592-605 [PMID: 21685204 DOI: 10.1093/hmg/ddr280]
54 **Doles J**, Hemann MT. Nek4 status differentially alters sensitivity to distinct microtubule poisons. *Cancer Res* 2010; **70:** 1033-1041 [PMID: 20103636 DOI: 10.1158/0008-5472/CAN-09-2113]
55 **Liu S**, Lu W, Obara T, Kuida S, Lehoczky J, Dewar K, Drummond IA, Beier DR. A defect in a novel Nek-family kinase causes cystic kidney disease in the mouse and in zebrafish. *Development* 2002; **129:** 5839-5846 [PMID: 12421721 DOI: 10.1242/dev.00173]
56 **Mahjoub MR**, Trapp ML, Quarmby LM. NIMA-related kinases defective in murine models of polycystic kidney diseases localize to primary cilia and centrosomes. *J Am Soc Nephrol* 2005; **16:** 3485-3489 [PMID: 16267153]
57 **Young CL**, Khoshnevis S, Karbstein K. Cofactor-dependent specificity of a DEAD-box protein. *Proc Natl Acad Sci USA* 2013; **110:** E2668-E2676 [PMID: 23630256 DOI: 10.1073/pnas.1302577110]
58 **Motose H**, Hamada T, Yoshimoto K, Murata T, Hasebe M, Watanabe Y, Hashimoto T, Sakai T, Takahashi T. NIMA-related kinases 6, 4, and 5 interact with each other to regulate microtubule organization during epidermal cell expansion in Arabidopsis thaliana. *Plant J* 2011; **67:** 993-1005 [PMID: 21605211 DOI: 10.1111/j.1365-313X.2011.04652.x]
59 **Vigneault F**, Lachance D, Cloutier M, Pelletier G, Levasseur C, Séguin A. Members of the plant NIMA-related kinases are involved in organ development and vascularization in poplar, Arabidopsis and rice. *Plant J* 2007; **51:** 575-588 [PMID: 17886359 DOI: 10.1111/j.1365-313X.2007.03161.x]
60 **Shimizu K**, Sawasaki T. Nek5, a novel substrate for caspase-3, promotes skeletal muscle differentiation by up-regulating caspase activity. *FEBS Lett* 2013; **587:** 2219-2225 [PMID: 23727203 DOI: 10.1016/j.febslet.2013.05.049]
61 **Larsen BD**, Rampalli S, Burns LE, Brunette S, Dilworth FJ, Megeney LA. Caspase 3/caspase-activated DNase promote cell differentiation by inducing DNA strand breaks. *Proc Natl Acad Sci USA* 2010; **107:** 4230-4235 [PMID: 20160104]
62 **Minoguchi S**, Minoguchi M, Yoshimura A. Differential control of the NIMA-related kinases, Nek6 and Nek7, by serum stimulation. *Biochem Biophys Res Commun* 2003; **301:** 899-906 [PMID: 12589797 DOI: 10.1016/S0006-291X(03)00049-4]
63 **Meirelles GV**, Silva JC, Mendonça Y de A, Ramos CH, Torriani IL, Kobarg J. Human Nek6 is a monomeric mostly globular kinase with an unfolded short N-terminal domain. *BMC Struct Biol* 2011; **11:** 12 [PMID: 21320329]
64 **Belham C**, Comb MJ, Avruch J. Identification of the NIMA family kinases NEK6/7 as regulators of the p70 ribosomal S6 kinase. *Curr Biol* 2001; **11:** 1155-1167 [PMID: 11516946 DOI: 10.1016/S0960-9822(01)00369-4]
65 **Lizcano JM**, Deak M, Morrice N, Kieloch A, Hastie CJ, Dong

L, Schutkowski M, Reimer U, Alessi DR. Molecular basis for the substrate specificity of NIMA-related kinase-6 (NEK6). Evidence that NEK6 does not phosphorylate the hydrophobic motif of ribosomal S6 protein kinase and serum- and glucocorticoid-induced protein kinase in vivo. *J Biol Chem* 2002; **277:** 27839-27849 [PMID: 12023960 DOI: 10.1074/jbc.M202042200]

66 **Roig J**, Mikhailov A, Belham C, Avruch J. Nercc1, a mammalian NIMA-family kinase, binds the Ran GTPase and regulates mitotic progression. *Genes Dev* 2002; **16:** 1640-1658 [PMID: 12101123 DOI: 10.1101/gad.972202]

67 **Belham C**, Roig J, Caldwell JA, Aoyama Y, Kemp BE, Comb M, Avruch J. A mitotic cascade of NIMA family kinases. Nercc1/Nek9 activates the Nek6 and Nek7 kinases. *J Biol Chem* 2003; **278:** 34897-34909 [PMID: 12840024 DOI: 10.1074/jbc.M303663200]

68 **Rapley J**, Nicolàs M, Groen A, Regué L, Bertran MT, Caelles C, Avruch J, Roig J. The NIMA-family kinase Nek6 phosphorylates the kinesin Eg5 at a novel site necessary for mitotic spindle formation. *J Cell Sci* 2008; **121:** 3912-3921 [PMID: 19001501 DOI: 10.1242/jcs.035360]

69 **Bertran MT**, Sdelci S, Regué L, Avruch J, Caelles C, Roig J. Nek9 is a Plk1-activated kinase that controls early centrosome separation through Nek6/7 and Eg5. *EMBO J* 2011; **30:** 2634-2647 [PMID: 21642957 DOI: 10.1038/emboj.2011.179]

70 **Sdelci S**, Bertran MT, Roig J. Nek9, Nek6, Nek7 and the separation of centrosomes. *Cell Cycle* 2011; **10:** 3816-3817 [PMID: 22064517 DOI: 10.4161/cc.10.22.18226]

71 **Matsuda A**, Suzuki Y, Honda G, Muramatsu S, Matsuzaki O, Nagano Y, Doi T, Shimotohno K, Harada T, Nishida E, Hayashi H, Sugano S. Large-scale identification and characterization of human genes that activate NF-kappaB and MAPK signaling pathways. *Oncogene* 2003; **22:** 3307-3318 [PMID: 12761501 DOI: 10.1038/sj.onc.1206406]

72 **Chen Y**, Chen PL, Chen CF, Jiang X, Riley DJ. Never-in-mitosis related kinase 1 functions in DNA damage response and checkpoint control. *Cell Cycle* 2008; **7:** 3194-3201 [PMID: 18843199 DOI: 10.4161/cc.7.20.6815]

73 **Melixetian M**, Klein DK, Sørensen CS, Helin K. NEK11 regulates CDC25A degradation and the IR-induced G2/M checkpoint. *Nat Cell Biol* 2009; **11:** 1247-1253 [PMID: 19734889 DOI: 10.1038/ncb1969]

74 **Moniz LS**, Stambolic V. Nek10 mediates G2/M cell cycle arrest and MEK autoactivation in response to UV irradiation. *Mol Cell Biol* 2011; **31:** 30-42 [PMID: 20956560 DOI: 10.1128/MCB.00648-10]

75 **Kang J**, Goodman B, Zheng Y, Tantin D. Dynamic regulation of Oct1 during mitosis by phosphorylation and ubiquitination. *PLoS One* 2011; **6:** e23872 [PMID: 21897860 DOI: 10.1371/journal.pone.0023872]

76 **Hashimoto Y**, Akita H, Hibino M, Kohri K, Nakanishi M. Identification and characterization of Nek6 protein kinase, a potential human homolog of NIMA histone H3 kinase. *Biochem Biophys Res Commun* 2002; **293:** 753-758 [PMID: 12054534 DOI: 10.1016/S0006-291X(02)00297-8]

77 **Skoblov M**, Marakhonov A, Marakasova E, Guskova A, Chandhoke V, Birerdinc A, Baranova A. Protein partners of KCTD proteins provide insights about their functional roles in cell differentiation and vertebrate development. *Bioessays* 2013; **35:** 586-596 [PMID: 23592240 DOI: 10.1002/bies.201300002]

78 **Lee EJ**, Hyun SH, Chun J, Kang SS. Human NIMA-related kinase 6 is one of the Fe65 WW domain binding proteins. *Biochem Biophys Res Commun* 2007; **358:** 783-788 [PMID: 17512906 DOI: 10.1016/j.bbrc.2007.04.203]

79 **Takeno A**, Takemasa I, Doki Y, Yamasaki M, Miyata H, Takiguchi S, Fujiwara Y, Matsubara K, Monden M. Integrative approach for differentially overexpressed genes in gastric cancer by combining large-scale gene expression profiling and network analysis. *Br J Cancer* 2008; **99:** 1307-1315 [PMID: 18827816 DOI: 10.1038/sj.bjc.6604682]

80 **Chen J**, Li L, Zhang Y, Yang H, Wei Y, Zhang L, Liu X, Yu L. Interaction of Pin1 with Nek6 and characterization of their expression correlation in Chinese hepatocellular carcinoma patients. *Biochem Biophys Res Commun* 2006; **341:** 1059-1065 [PMID: 16476580 DOI: 10.1016/j.bbrc.2005.12.228]

81 **Jee HJ**, Kim HJ, Kim AJ, Song N, Kim M, Lee HJ, Yun J. The inhibition of Nek6 function sensitizes human cancer cells to premature senescence upon serum reduction or anticancer drug treatment. *Cancer Lett* 2013; **335:** 175-182 [PMID: 23416273 DOI: 10.1016/j.canlet.2013.02.012]

82 **Capra M**, Nuciforo PG, Confalonieri S, Quarto M, Bianchi M, Nebuloni M, Boldorini R, Pallotti F, Viale G, Gishizky ML, Draetta GF, Di Fiore PP. Frequent alterations in the expression of serine/threonine kinases in human cancers. *Cancer Res* 2006; **66:** 8147-8154 [PMID: 16912193 DOI: 10.1158/0008-5472.CAN-05-3489]

83 **Kasap E**, Boyacioglu SO, Korkmaz M, Yuksel ES, Unsal B, Kahraman E, Ozütemiz O, Yuceyar H. Aurora kinase A (AURKA) and never in mitosis gene A-related kinase 6 (NEK6) genes are upregulated in erosive esophagitis and esophageal adenocarcinoma. *Exp Ther Med* 2012; **4:** 33-42 [PMID: 23060919 DOI: 10.3892/etm.2012.561]

84 **Nassirpour R**, Shao L, Flanagan P, Abrams T, Jallal B, Smeal T, Yin MJ. Nek6 mediates human cancer cell transformation and is a potential cancer therapeutic target. *Mol Cancer Res* 2010; **8:** 717-728 [PMID: 20407017 DOI: 10.1158/1541-7786.MCR-09-0291]

85 **Jeon YJ**, Lee KY, Cho YY, Pugliese A, Kim HG, Jeong CH, Bode AM, Dong Z. Role of NEK6 in tumor promoter-induced transformation in JB6 C141 mouse skin epidermal cells. *J Biol Chem* 2010; **285:** 28126-28133 [PMID: 20595392 DOI: 10.1074/jbc.M110.137190]

86 **Jee HJ**, Kim AJ, Song N, Kim HJ, Kim M, Koh H, Yun J. Nek6 overexpression antagonizes p53-induced senescence in human cancer cells. *Cell Cycle* 2010; **9:** 4703-4710 [PMID: 21099361 DOI: 10.4161/cc.9.23.14059]

87 **Jee HJ**, Kim HJ, Kim AJ, Song N, Kim M, Yun J. Nek6 suppresses the premature senescence of human cancer cells induced by camptothecin and doxorubicin treatment. *Biochem Biophys Res Commun* 2011; **408:** 669-673 [PMID: 21539811 DOI: 10.1016/j.bbrc.2011.04.083]

88 **Kim S**, Lee K, Rhee K. NEK7 is a centrosomal kinase critical for microtubule nucleation. *Biochem Biophys Res Commun* 2007; **360:** 56-62 [PMID: 17586473 DOI: 10.1016/j.bbrc.2007.05.206]

89 **Yissachar N**, Salem H, Tennenbaum T, Motro B. Nek7 kinase is enriched at the centrosome, and is required for proper spindle assembly and mitotic progression. *FEBS Lett* 2006; **580:** 6489-6495 [PMID: 17101132 DOI: 10.1016/j.febslet.2006.10.069]

90 **Cohen S**, Aizer A, Shav-Tal Y, Yanai A, Motro B. Nek7 kinase accelerates microtubule dynamic instability. *Biochim Biophys Acta* 2013; **1833:** 1104-1113 [PMID: 23313050 DOI: 10.1016/j.bbamcr.2012.12.021]

91 **Kim S**, Kim S, Rhee K. NEK7 is essential for centriole duplication and centrosomal accumulation of pericentriolar material proteins in interphase cells. *J Cell Sci* 2011; **124:** 3760-3770 [PMID: 22100915 DOI: 10.1242/jcs.078089]

92 **Richards MW**, O'Regan L, Mas-Droux C, Blot JM, Cheung J, Hoelder S, Fry AM, Bayliss R. An autoinhibitory tyrosine motif in the cell-cycle-regulated Nek7 kinase is released through binding of Nek9. *Mol Cell* 2009; **36:** 560-570 [PMID: 19941817 DOI: 10.1016/j.molcel.2009.09.038]

93 **Wang R**, Song Y, Xu X, Wu Q, Liu C. The expression of Nek7, FoxM1, and Plk1 in gallbladder cancer and their relationships to clinicopathologic features and survival. *Clin Transl Oncol* 2013; **15:** 626-632 [PMID: 23359173 DOI: 10.1007/s12094-012-0978-9]

94 **Bowers AJ**, Boylan JF. Nek8, a NIMA family kinase mem-

ber, is overexpressed in primary human breast tumors. *Gene* 2004; **328**: 135-142 [PMID: 15019993 DOI: 10.1016/j.gene.2003.12.002]

95 **Zalli D**, Bayliss R, Fry AM. The Nek8 protein kinase, mutated in the human cystic kidney disease nephronophthisis, is both activated and degraded during ciliogenesis. *Human Molecular Genetics* 2012; **21**: 1155-1171 [DOI: 10.1093/hmg/ddr544]

96 **Valkova N**, Yunis R, Mak SK, Kang K, Kultz D. Nek8 Mutation Causes Overexpression of Galectin-1, Sorcin, and Vimentin and Accumulation of the Major Urinary Protein in Renal Cysts of jck Mice. *Molecular Cellular Proteomics* 2005; **4**: 1007-1009 [DOI: 10.1074/mcp.M500091 MCP200]

97 **Sohara E**, Luo Y, Zhang J, Manning DK, Beier DR, Zhou J. Nek8 Regulates the Expression and Localization of Polycystin-1 and Polycystin-2. *J Am Soc Nephrol* 2008; **19**: 469-476 [DOI: 10.1681/ASN.2006090985]

98 **Roig J**, Groen A, Caldwell J, Avruch J. Active Nercc1 protein kinase concentrates at centrosomes early in mitosis and is necessary for proper spindle assembly. *Mol Biol Cell* 2005; **16**: 4827-4840 [PMID: 16079175 DOI: 10.1091/mbc.E05-04-0315]

99 **Holland PM**, Milne A, Garka K, Johnson RS, Willis C, Sims JE, Rauch CT, Bird TA, Virca GD. Purification, cloning, and characterization of Nek8, a novel NIMA-related kinase, and its candidate substrate Bicd2. *J Biol Chem* 2002; **277**: 16229-16240 [PMID: 11864968 DOI: 10.1074/jbc.M108662200]

100 **Sdelci S**, Schütz M, Pinyol R, Bertran MT, Regué L, Caelles C, Vernos I, Roig J. Nek9 phosphorylation of NEDD1/GCP-WD contributes to Plk1 control of γ-tubulin recruitment to the mitotic centrosome. *Curr Biol* 2012; **22**: 1516-1523 [PMID: 22818914 DOI: 10.1016/j.cub.2012.06.027]

101 **Regué L**, Sdelci S, Bertran MT, Caelles C, Reverter D, Roig J. DYNLL/LC8 protein controls signal transduction through the Nek9/Nek6 signaling module by regulating Nek6 binding to Nek9. *J Biol Chem* 2011; **286**: 18118-18129 [PMID: 21454704 DOI: 10.1074/jbc.M110.209080]

102 **Jackman M**, Lindon C, Nigg EA, Pines J. Active cyclin B1-Cdk1 first appears on centrosomes in prophase. *Nat Cell Biol* 2003; **5**: 143-148 [PMID: 12524548 DOI: 10.1038/ncb918]

103 **Wu Z**, Doondeea JB, Gholami AM, Janning MC, Lemeer S, Kramer K, Eccles SA, Gollin SM, Grenman R, Walch A, Feller SM, Kuster B. Quantitative chemical proteomics reveals new potential drug targets in head and neck cancer. *Mol Cell Proteomics* 2011; **10**: M111.011635 [PMID: 21955398 DOI: 10.1074/mcp.M111.011635]

104 **Boly R**, Gras T, Lamkami T, Guissou P, Serteyn D, Kiss R, Dubois J. Quercetin inhibits a large panel of kinases implicated in cancer cell biology. *Int J Oncol* 2011; **38**: 833-842 [PMID: 21206969 DOI: 10.3892/ijo.2010.890]

105 **Cooper MJ**, Cox NJ, Zimmerman EI, Dewar BJ, Duncan JS, Whittle MC, Nguyen TA, Jones LS, Ghose Roy S, Smalley DM, Kuan PF, Richards KL, Christopherson RI, Jin J, Frye SV, Johnson GL, Baldwin AS, Graves LM. Application of multiplexed kinase inhibitor beads to study kinome adaptations in drug-resistant leukemia. *PLoS One* 2013; **8**: e66755 [PMID: 23826126 DOI: 10.1371/journal.pone.0066755]

106 **Kaneta Y**, Ullrich A. NEK9 depletion induces catastrophic mitosis by impairment of mitotic checkpoint control and spindle dynamics. *Biochem Biophys Res Commun* 2013; **442**: 139-146 [PMID: 23665325 DOI: 10.1016/j.bbrc.2013.04.105]

107 **Huber AH**, Nelson WJ, Weis WI. Three-dimensional structure of the armadillo repeat region of beta-catenin. *Cell* 1997; **90**: 871-882 [PMID: 9298899 DOI: 10.1016/S0092-8674(00)80352-9]

108 **Antoniou AC**, Beesley J, McGuffog L, Sinilnikova OM, Healey S, Neuhausen SL, Ding YC, Rebbeck TR, Weitzel JN, Lynch HT, Isaacs C, Ganz PA, Tomlinson G, Olopade OI, Couch FJ, Wang X, Lindor NM, Pankratz VS, Radice P, Manoukian S, Peissel B, Zaffaroni D, Barile M, Viel A, Allavena A, Dall'Olio V, Peterlongo P, Szabo CI, Zikan M, Claes K, Poppe B, Foretova L, Mai PL, Greene MH, Rennert G, Lejbkowicz F, Glendon G, Ozcelik H, Andrulis IL, Thomassen M, Gerdes AM, Sunde L, Cruger D, Birk Jensen U, Caligo M, Friedman E, Kaufman B, Laitman Y, Milgrom R, Dubrovsky M, Cohen S, Borg A, Jernström H, Lindblom A, Rantala J, Stenmark-Askmalm M, Melin B, Nathanson K, Domchek S, Jakubowska A, Lubinski J, Huzarski T, Osorio A, Lasa A, Durán M, Tejada MI, Godino J, Benitez J, Hamann U, Kriege M, Hoogerbrugge N, van der Luijt RB, van Asperen CJ, Devilee P, Meijers-Heijboer EJ, Blok MJ, Aalfs CM, Hogervorst F, Rookus M, Cook M, Oliver C, Frost D, Conroy D, Evans DG, Lalloo F, Pichert G, Davidson R, Cole T, Cook J, Paterson J, Hodgson S, Morrison PJ, Porteous ME, Walker L, Kennedy MJ, Dorkins H, Peock S, Godwin AK, Stoppa-Lyonnet D, de Pauw A, Mazoyer S, Bonadona V, Lasset C, Dreyfus H, Leroux D, Hardouin A, Berthet P, Faivre L, Loustalot C, Noguchi T, Sobol H, Rouleau E, Nogues C, Frénay M, Vénat-Bouvet L, Hopper JL, Daly MB, Terry MB, John EM, Buys SS, Yassin Y, Miron A, Goldgar D, Singer CF, Dressler AC, Gschwantler-Kaulich D, Pfeiler G, Hansen TV, Jønson L, Agnarsson BA, Kirchhoff T, Offit K, Devlin V, Dutra-Clarke A, Piedmonte M, Rodriguez GC, Wakeley K, Boggess JF, Basil J, Schwartz PE, Blank SV, Toland AE, Montagna M, Casella C, Imyanitov E, Tihomirova L, Blanco I, Lazaro C, Ramus SJ, Sucheston L, Karlan BY, Gross J, Schmutzler R, Wappenschmidt B, Engel C, Meindl A, Lochmann M, Arnold N, Heidemann S, Varon-Mateeva R, Niederacher D, Sutter C, Deissler H, Gadzicki D, Preisler-Adams S, Kast K, Schönbuchner I, Caldes T, de la Hoya M, Aittomäki K, Nevanlinna H, Simard J, Spurdle AB, Holland H, Chen X, Platte R, Chenevix-Trench G, Easton DF. Common breast cancer susceptibility alleles and the risk of breast cancer for BRCA1 and BRCA2 mutation carriers: implications for risk prediction. *Cancer Res* 2010; **70**: 9742-9754 [PMID: 21118973 DOI: 10.1158/0008-5472]

109 **Ahmed S**, Thomas G, Ghoussaini M, Healey CS, Humphreys MK, Platte R, Morrison J, Maranian M, Pooley KA, Luben R, Eccles D, Evans DG, Fletcher O, Johnson N, dos Santos Silva I, Peto J, Stratton MR, Rahman N, Jacobs K, Prentice R, Anderson GL, Rajkovic A, Curb JD, Ziegler RG, Berg CD, Buys SS, McCarty CA, Feigelson HS, Calle EE, Thun MJ, Diver WR, Bojesen S, Nordestgaard BG, Flyger H, Dörk T, Schürmann P, Hillemanns P, Karstens JH, Bogdanova NV, Antonenkova NN, Zalutsky IV, Bermisheva M, Fedorova S, Khusnutdinova E, Kang D, Yoo KY, Noh DY, Ahn SH, Devilee P, van Asperen CJ, Tollenaar RA, Seynaeve C, Garcia-Closas M, Lissowska J, Brinton L, Peplonska B, Nevanlinna H, Heikkinen T, Aittomäki K, Blomqvist C, Hopper JL, Southey MC, Smith L, Spurdle AB, Schmidt MK, Broeks A, van Hien RR, Cornelissen S, Milne RL, Ribas G, González-Neira A, Benitez J, Schmutzler RK, Burwinkel B, Bartram CR, Meindl A, Brauch H, Justenhoven C, Hamann U, Chang-Claude J, Hein R, Wang-Gohrke S, Lindblom A, Margolin S, Mannermaa A, Kosma VM, Kataja V, Olson JE, Wang X, Fredericksen Z, Giles GG, Severi G, Baglietto L, English DR, Hankinson SE, Cox DG, Kraft P, Vatten LJ, Hveem K, Kumle M, Sigurdson A, Doody M, Bhatti P, Alexander BH, Hooning MJ, van den Ouweland AM, Oldenburg RA, Schutte M, Hall P, Czene K, Liu J, Li Y, Cox A, Elliott G, Brock I, Reed MW, Shen CY, Yu JC, Hsu GC, Chen ST, Anton-Culver H, Ziogas A, Andrulis IL, Knight JA, Beesley J, Goode EL, Couch F, Chenevix-Trench G, Hoover RN, Ponder BA, Hunter DJ, Pharoah PD, Dunning AM, Chanock SJ, Easton DF. Newly discovered breast cancer susceptibility loci on 3p24 and 17q23.2. *Nat Genet* 2009; **41**: 585-590 [PMID: 19330027 DOI: 10.1038/ng.354]

110 **Mulligan AM**, Couch FJ, Barrowdale D, Domchek SM, Eccles D, Nevanlinna H, Ramus SJ, Robson M, Sherman M, Spurdle AB, Wappenschmidt B, Lee A, McGuffog L, Healey

S, Sinilnikova OM, Janavicius R, Hansen Tv, Nielsen FC, Ejlertsen B, Osorio A, Muñoz-Repeto I, Durán M, Godino J, Pertesi M, Benítez J, Peterlongo P, Manoukian S, Peissel B, Zaffaroni D, Cattaneo E, Bonanni B, Viel A, Pasini B, Papi L, Ottini L, Savarese A, Bernard L, Radice P, Hamann U, Verheus M, Meijers-Heijboer HE, Wijnen J, Gómez García EB, Nelen MR, Kets CM, Seynaeve C, Tilanus-Linthorst MM, van der Luijt RB, van Os T, Rookus M, Frost D, Jones JL, Evans DG, Lalloo F, Eeles R, Izatt L, Adlard J, Davidson R, Cook J, Donaldson A, Dorkins H, Gregory H, Eason J, Houghton C, Barwell J, Side LE, McCann E, Murray A, Peock S, Godwin AK, Schmutzler RK, Rhiem K, Engel C, Meindl A, Ruehl I, Arnold N, Niederacher D, Sutter C, Deissler H, Gadzicki D, Kast K, Preisler-Adams S, Varon-Mateeva R, Schoenbuchner I, Fiebig B, Heinritz W, Schäfer D, Gevensleben H, Caux-Moncoutier V, Fassy-Colcombet M, Cornelis F, Mazoyer S, Léoné M, Boutry-Kryza N, Hardouin A, Berthet P, Muller D, Fricker JP, Mortemousque I, Pujol P, Coupier I, Lebrun M, Kientz C, Longy M, Sevenet N, Stoppa-Lyonnet D, Isaacs C, Caldes T, de la Hoya M, Heikkinen T, Aittomäki K, Blanco I, Lazaro C, Barkardottir RB, Soucy P, Dumont M, Simard J, Montagna M, Tognazzo S, D'Andrea E, Fox S, Yan M, Rebbeck T, Olopade O, Weitzel JN, Lynch HT, Ganz PA, Tomlinson GE, Wang X, Fredericksen Z, Pankratz VS, Lindor NM, Szabo C, Offit K, Sakr R, Gaudet M, Bhatia J, Kauff N, Singer CF, Tea MK, Gschwantler-Kaulich D, Fink-Retter A, Mai PL, Greene MH, Imyanitov E, O'Malley FP, Ozcelik H, Glendon G, Toland AE, Gerdes AM, Thomassen M, Kruse TA, Jensen UB, Skytte AB, Caligo MA, Soller M, Henriksson K, Wachenfeldt vA, Arver B, Stenmark-Askmalm M, Karlsson P, Ding YC, Neuhausen SL, Beattie M, Pharoah PD, Moysich KB, Nathanson KL, Karlan BY, Gross J, John EM, Daly MB, Buys SM, Southey MC, Hopper JL, Terry MB, Chung W, Miron AF, Goldgar D, Chenevix-Trench G, Easton DF, Andrulis IL, Antoniou AC. Common breast cancer susceptibility alleles are associated with tumour subtypes in BRCA1 and BRCA2 mutation carriers: results from the Consortium of Investigators of Modifiers of BRCA1/2. *Breast Cancer Res* 2011; **13**: R110 [PMID: 22053997 DOI: 10.1186/bcr3052.]

111 **Noguchi K**, Fukazawa H, Murakami Y, Uehara Y. Nek11, a new member of the NIMA family of kinases, involved in DNA replication and genotoxic stress responses. *J Biol Chem* 2002; **277**: 39655-39665 [PMID: 12154088]

112 **Noguchi K**, Fukazawa H, Murakami Y, Uehara Y. Nucleolar Nek11 is a novel target of Nek2A in G1/S-arrested cells. *J Biol Chem* 2004; **279**: 32716-32727 [PMID: 15161910]

113 **Alborghetti MR**, Furlan AS, Kobarg J. FEZ2 has acquired additional protein interaction partners relative to FEZ1: functional and evolutionary implications. *PLoS One* 2011; **6**: e17426 [PMID: 21408165 DOI: 10.1371/journal.pone.0017426]

114 **Lanza DC**, Meirelles GV, Alborghetti MR, Abrile CH, Lenz G, Kobarg J.FEZ1 interacts with CLASP2 and NEK1 through coiled-coil regions and their cellular colocalization suggests centrosomal functions and regulation by PKC. *Mol Cell Biochem* 2010; **338**: 35-45 [PMID: 19924516 DOI: 10.1007/s11010-009-0317-9]

115 **Hong Z**, Jiang J, Lan L, Nakajima S, Kanno S, Koseki H, Yasui A. A polycomb group protein, PHF1, is involved in the response to DNA double-strand breaks in human cell. *Nucleic Acids Res* 2008; **36**: 2939-2947 [PMID: 18385154 DOI: 10.1093/nar/gkn146]

116 **Sonoda E**, Matsusaka T, Morrison C, Vagnarelli P, Hoshi O, Ushiki T, Nojima K, Fukagawa T, Waizenegger IC, Peters JM, Earnshaw WC, Takeda S. Scc1/Rad21/Mcd1 is required for sister chromatid cohesion and kinetochore function in vertebrate cells. *Dev Cell* 2001; **1**: 759-770 [PMID: 11740938 DOI: 10.1016/S1534-5807(01)00088-0]

117 **Ishizaka A**, Mizutani T, Kobayashi K, Tando T, Sakurai K, Fujiwara T, Iba H. Double plant homeodomain (PHD) finger proteins DPF3a and -3b are required as transcriptional co-activators in SWI/SNF complex-dependent activation of NF-κB RelA/p50 heterodimer. *J Biol Chem* 2012; **287**: 11924-11933 [PMID: 22334708 DOI: 10.1074/jbc.M111.322792]

118 **Ewing RM**, Chu P, Elisma F, Li H, Taylor P, Climie S, McBroom-Cerajewski L, Robinson MD, O'Connor L, Li M, Taylor R, Dharsee M, Ho Y, Heilbut A, Moore L, Zhang S, Ornatsky O, Bukhman YV, Ethier M, Sheng Y, Vasilescu J, Abu-Farha M, Lambert JP, Duewel HS, Stewart II, Kuehl B, Hogue K, Colwill K, Gladwish K, Muskat B, Kinach R, Adams SL, Moran MF, Morin GB, Topaloglou T, Figeys D. Large-scale mapping of human protein-protein interactions by mass spectrometry. *Mol Syst Biol* 2007; **3**: 89 [PMID: 17353931 DOI: 10.1038/msb4100134]

119 **Villumsen BH**, Danielsen JR, Povlsen L, Sylvestersen KB, Merdes A, Beli P, Yang YG, Choudhary C, Nielsen ML, Mailand N, Bekker-Jensen S. A new cellular stress response that triggers centriolar satellite reorganization and ciliogenesis. *EMBO J* 2013; **32**: 3029-3040 [PMID: 24121310 DOI: 10.1038/emboj.2013.223]

120 **Chaki M**, Airik R, Ghosh AK, Giles RH, Chen R, Slaats GG, Wang H, Hurd TW, Zhou W, Cluckey A, Gee HY, Ramaswami G, Hong CJ, Hamilton BA, Cervenka I, Ganji RS, Bryja V, Arts HH, van Reeuwijk J, Oud MM, Letteboer SJ, Roepman R, Husson H, Ibraghimov-Beskrovnaya O, Yasunaga T, Walz G, Eley L, Sayer JA, Schermer B, Liebau MC, Benzing T, Le Corre S, Drummond I, Janssen S, Allen SJ, Natarajan S, O'Toole JF, Attanasio M, Saunier S, Antignac C, Koenekoop RK, Ren H, Lopez I, Nayir A, Stoetzel C, Dollfus H, Massoudi R, Gleeson JG, Andreoli SP, Doherty DG, Lindstrad A, Golzio C, Katsanis N, Pape L, Abboud EB, Al-Rajhi AA, Lewis RA, Omran H, Lee EY, Wang S, Sekiguchi JM, Saunders R, Johnson CA, Garner E, Vanselow K, Andersen JS, Shlomai J, Nurnberg G, Nurnberg P, Levy S, Smogorzewska A, Otto EA, Hildebrandt F. Exome capture reveals ZNF423 and CEP164 mutations, linking renal ciliopathies to DNA damage response signaling. *Cell* 2012; **150**: 533-548 [PMID: 22863007 DOI: 10.1016/j.cell.2012.06.028]

121 **Zhu YY**, Lan JP, Yu J.Interaction between a novel centrosomal protein TACP1 and mitotic kinase Nek2A [Article in Chinese]. *Zhejiang Daxue Xuebao Yixueban* 2007; **36**: 337-42 [PMID: 17717823]

122 **Prime G**, Markie D. The telomere repeat binding protein Trf1 interacts with the spindle checkpoint protein Mad1 and Nek2 mitotic kinase. *Cell Cycle* 2005; **4**: 121-124 [PMID: 15611654]

123 **Lou Y**, Xie W, Zhang DF, Yao JH, Luo ZF, Wang YZ, Shi YY, Yao XB. Nek2A specifies the centrosomal localization of Erk2. *Biochem Biophys Res Commun* 2004; **321**: 495-501 [PMID: 15358203]

124 **Helps NR**, Luo X, Barker HM, Cohen PT. NIMA-related kinase 2 (Nek2), a cell-cycle-regulated protein kinase localized to centrosomes, is complexed to protein phosphatase 1. *Biochem J* 2000; **349**: 509-518 [PMID: 10880350]

125 **Ashburner M**, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 2000; **25**: 25-29 [PMID: 10802651 DOI: 10.1038/75556]

126 **Shannon P**, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 2003; **13**: 2498-2504 [PMID: 14597658]

127 **Mi J**, Guo C, Brautigan DL, Larner JM. Protein phosphatase-1alpha regulates centrosome splitting through Nek2. *Cancer Res* 2007; **67**: 1082-1089 [PMID: 17283141 DOI:

10.1158/0008-5472.CAN-06-3071]
128 **Sann S**, Wang Z, Brown H, Jin Y. Roles of endosomal trafficking in neurite outgrowth and guidance. *Trends Cell Biol* 2009; **19**: 317-324 [PMID: 19540123 DOI: 10.1016/j.tcb.2009.05.001]
129 **Pelengaris S**, Khan M. The Molecular Biology of Cancer: A Bridge from Bench to Bedside. 2nd ed. Oxford: Wiley-Blackwell, 2013: 324

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Artigo II

Nek5 interacts with mitochondrial proteins and interferes negatively in mitochondrial mediated cell death and respiration

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Nek5 interacts with mitochondrial proteins and interferes negatively in mitochondrial mediated cell death and respiration ☆

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ABSTRACT

Mitochondria are involved in energy supply, signaling, cell death and cellular differentiation and have been implicated in several human diseases. Neks (NIMA-related kinases) represent a family of mammal protein kinases that play essential roles in cell-cycle progression, but other functions have recently been related. A yeast two-hybrid (Y2H) screen was performed to identify and characterize Nek5 interaction partners and the mitochondrial proteins Cox11, MTX-2 and BCLAF1 were retrieved. Apoptosis assay showed protective effects of stable hNek5 expression from Hek293-T's cell death after thapsigargin treatment (2 μM). Nek5 silenced cells as well as cells expressing a "kinase dead" version of Nek5, displayed an increase in ROS formation after 4 h of thapsigargin treatment. Mitochondrial respiratory chain activity was found decreased upon stable hNek5expression. Cells silenced for hNek5 on the other hand presented 1.7 fold increased basal rates of respiration, especially at the electrons transfer steps from TMPD to cytochrome c and at the complex II. In conclusion, our data suggest for the first time mitochondrial localization and functions for Nek5 and its participation in cell death and cell respiration regulation. Stable expression of h*Nek5* in Hek293T cells resulted in enhanced cell viability, decreased cell death and drug resistance, while depletion of hNek5by shRNA overcame cancer cell drug resistance and induced apoptosis *in vitro*. Stable expression of hNek5 also inhibits thapsigargin promoted apoptosis and the respiratory chain complex IV in HEK293T cells.

1. Introduction

Mitochondria are organelles responsible for the generation of the cell energy through formation of adenosine triphosphate (ATP) [1]. Besides supplying cellular energy, they are involved in the regulation of cell death, cellular differentiation, calcium homeostasis and the control of the cell cycle and have been implicated in several human diseases including endocrine related disorders such as diabetes mellitus [2–4].

Proteins that change localization inside the cells and undergo post-translational modifications become candidates to connect biological processes with proximal signal transduction. Most of the pro-death BCL-2 members demonstrate modifications and protein translocation upon receipt of a death stimulus [5]. Many other processes occur inside or dependent of mitochondria. For example, NADH and FADH2 are generated during glycolysis, fatty acid oxidation and the citric acid cycle, which are oxidized within the mitochondria. Electron transfer through a series of respiratory complexes ends with the terminal reduction of molecular oxygen to water. Four respiratory complexes designated Complex I (NADH dehydrogenase), Complex II (succinate dehydrogenase), Complex III (ubiquinol cytochrome c reductase) and Complex IV (cytochrome c oxidase). These respiratory complexes are enriched in segments of the inner mitochondrial membrane that invaginate into cristae [6].

A genetic screen in the filamentous fungus *Aspergillus nidulans*, identified Never In Mitosis A (NIMA) as a temperature sensitive mutants that failed in cell cycle progression [7]. Osmani and co-workers, characterized the NIMA gene to encode a serine/threonine protein kinase

☆ Author contributions: TDMH, PFP, PGG, AEV and JK performed the literature search, data analysis and contributed specific parts of the manuscript. TDMH performed the experiments with help from PFP and PGG. TDMH and JK elaborate the final version of the text and JK supervised the project. All authors read and approved the final version.

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[1] In memoriam.

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localized to the spindle pole body that plays essential roles in cell-cycle progression [8,9]. The Neks (NIMA-related kinases) represent a family of mammalian protein kinases that consists of 11 members that share 40–45% identity with NIMA in the catalytic domain. The usually C-terminal located regulatory domain and has greater variability and is involved in determining the substrate specificity and interaction with other proteins [10]. All 11 human Neks have been functionally assigned to one of the three core functions in mammals, established for this family: 1) centrioles/mitosis, 2) primary ciliary function/ciliopathies and 3) the DNA damage response (DDR) [11]. Besides that, a new function has been associated recently to the Nek family. Chen and co-workers demonstrated that Nek1 acts in apoptosis signaling, specifically by phosphorylation of the voltage-dependent anion-selective channel protein 1 (VDAC1) [12]. It is a pore complex that functions both as a voltage dependent anion channel and permeability pore that regulates cytochrome c leakage to the cytoplasm, which upon exit initiates apoptosis. Upon apoptotic stimuli Nek1 is degraded and the lack of VDAC1 phosphorylation causes opening of its channel, loss of membrane potential and cytochrome c leakage to the cytoplasm [12].

Among the members of the Nek family, human Nek5 (hNek5) is the kinase with the smallest amount of information. Little is known about its function and subcellular localization. Nek5 is capable to interact with caspase-3 and this interaction is important for skeletal muscle differentiation [13]. Caspase-3 is a protease involved in mechanisms such as apoptosis and cell differentiation. The functional interaction of Nek5 with caspase-3 in cell differentiation, suggests that its involvement in cell death should be also explored. Together with the profile of mitochondrial proteins identified here as interactors of Nek5 we decided to investigate the role of hNek5 in cell death and cell respiration. Our data suggest mitochondrial localization for hNek5 and its role in the prevention of cell death.

2. Materials and methods

2.1. Yeast two-hybrid screening

The cDNA encoding the C-terminus of hNek5 (a.a. 260–708) was cloned in the pGBKT7 vector to be used as bait against a human universal cDNA library. A yeast two-hybrid screen was performed following the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech Laboratories, Inc., Mountain View, CA) according to the manufacturer's instructions. Interactors from the screen were detected by growth of co-transformed yeast cells in a selective media which is absent of tryptophan, leucine, adenine and histidine (−W, −L, −A, −H) but containing aureobasidin antibiotic and X-α-Gal to promote a blue color to the colony as the reporters are activated by the interaction. In order to identify the "preys" genes, the extracted DNAs were sequenced and analyzed at the Integrated Interactome System (IIS) platform, developed at National Laboratory of Biosciences, Brazil (http://www.lge.ibi.unicamp.br/lnbio/IIS/) [14].

2.2. Immunofluorescence and confocal microscopy

Cells grown on coverslips to 60% confluence were fixed in ice-cold methanol. Fixed cells were permeabilized with 0.5% Triton X-100 and blocked with 3% BSA and 0.1% Triton X-100 in PBS. Primary antibodies included anti-Nek5 (1:50; Santa Cruz Biotechnology, Dallas, USA), anti-BCLAF1 (1:50; Santa Cruz Biotechnology), anti-Cox11 (1:25; Novus Biologicals, Littleton, USA), anti-MTX2 (1:25; Novus Biologicals), anti-VDAC (1:50; Abcam, Cambridge, UK) and Mitotracker Deep Red (500 nM; Life Technologies, Carlsbad, USA). Subsequently, fluorescent-labeled secondary antibodies were incubated at a dilution of 1:200, including anti-rabbit IgG-Alexa 594 (red; Molecular Probes, Eugene, OR, USA) and anti-mouse or anti-goat IgG-Alexa 488 (green; Molecular Probes, Eugene, OR, USA). Hoechst dye was used for nuclei staining. Samples were mounted in ProLong® Gold Antifade Reagent (Life Technologies Corporation, Carlsbad, CA) and images were acquired using confocal microscope Leica TCS SP8 from the National Biosciences Laboratory — LNBio/CNPEM and confocal microscopy Zeiss LSM 510 confocal laser-scanning microscope from the National Institute of Photonics Applied to Cell Biology/UNICAMP. Images were processed using LAS AF (Leica) and LSM 5 Image Examiner (Carl Zeiss) software.

2.3. Cell culture and generation of stable cell lines

Adherent HEK293T, T-REx-293 and U2-OS cells were obtained from ATCC and maintained at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium, DMEM (Gibco), supplemented with 10% certified fetal bovine serum (Gibco) and penicillin/streptomycin (100 units/ml, Gibco). Specifically, 5 mg/ml blasticidin was added to T-REx-293 culture. To generate Nek5 stable cell lines, T-REx-293 cells were transfected with the empty vector pcDNATM5/FRT/TO/FLAG and pcDNATM5/FRT/TO/FLAG-Nek5 using Lipofectamine 2000 according to the manufacturer's protocol (Life Technologies). After transfection, cells were transferred into fresh DMEM containing 50 μg/ml hygromycin B (Invitrogen, Carlsbad, CA) and the medium was replaced every 3 days. Several colonies were separately expanded, induced with 2 mg/ml tetracycline for 48 h and analyzed by immunoprecipitation and immunoblotting.

2.4. Generation of Nek5 depleted cells

Nek5 shRNA (h) Lentiviral Particles (Santa Cruz Biotechnology, Inc.) were used to knock down hNek5 as previously reported by the company. Briefly, 5.0×10^4 infectious units of virus of the lentiviral particles were added to the Hek293T cells, followed by a clone selection with 3 μg/ml puromycin. Several colonies were picked, expanded and assayed for stable shRNA expression by RT-PCR. The PCR pairs of primer sequences for Nek5 were sense 5′ GGAATTCGAGAATCTTATTCCCAAAT ATTT 3′ and antisense 5′ AATCTCGAGGTCGACTCACATCAGGATGAGCAC 3′, and those for α-tubulin were sense 5′ GGATCCAAGCTTAGATGCGT GAGTGCATCTCCATCC 3′ and antisense 5′ CTGCAGGTCGACTTAGTATT CCTCTCCTTCTTCCTC 3′.

2.5. Mitochondria isolation

Mitochondria were isolated using the Qproteome® Mitochondria Isolation Handbook (Qiagen) according to the manufacturer's instructions. Briefly, 2×10^7 of stably transfected Nek5 knocked down cells were trypsinized, washed with PBS and 0.9% of NaCl and centrifuged at 600 ×g, 4 °C for 5 min. The precipitate was suspended in mitochondria lysis buffer on ice for 10 min, centrifuged at 1000 ×g for 10 min, 4 °C and the supernatant collected as the cytosolic fraction. To extract high pure mitochondria, the pellet was suspended in disruption buffer and this homogenate was centrifuged at 1.000 ×g at 4 °C for 10 min. Then, the supernatant was centrifuged in a new tube at 6.000 ×g at 4 °C for 10 min and the precipitate from this process represents the isolated mitochondria. Protein extract from the mitochondria fraction was suspended as previously described.

2.6. Immunoprecipitation

IP procedures were performed at 4 °C with Hek293T and hNek5 stably transfected-cells that were lysed in ERB buffer (50 mM Tris, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 10% glycerol and 0,2% NP40) with protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) and centrifuged at 20.000 ×g for 30 min at 4 °C. The supernatants were used for immunoprecipitation with protein G sepharose slurry (20 μl), which had been washed twice with dilution buffer (DB: 10 mM Tris, 150 mM NaCl and 0,5 mM EDTA) and were then incubated with 5 μg Nek5 or BCLAF1 antibody for 2 h with DB at 4 °C on a mixer. For FLAG immunoprecipitation we used the ANTI-FLAG(R) M2 Affinity

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Gel washed three times with DB. The proteins in the supernatant were incubated with the resin (FLAG or with BCLAf1 and Nek5 antibody) overnight at 4 °C on a mixer. The supernatant was discarded and the beads were washed five times with 1000 μl DF, followed by incubation with Laemmli buffer (90 mM DTT, 2% SDS, 80 mM Tris and 10% glycerol) at 100 °C for 10 min. The supernatants were separated by SDS-PAGE.

2.7. Immunoblotting analysis

The Bradford Method was used to determine the protein concentration of the extracts from mitochondria isolation [15]. Thus, 50 μg of protein was separated by SDS-PAGE and blotted onto Immobilon-P membrane (Millipore Corp., Bedford, MA). The protein bands were probed by using the proper primary antibodies in 3% bovine serum albumin blocking solution: anti-VDAC (dilution: 1:500), anti-β-Tub (1:1000) and anti-Cox11 (1:750) were obtained from Abcam; anti-Nek5 (1:500), anti-BCLAF1 (1:1000), anti-GADPH (1:500) and anti-lamin A (1:500) from Santa Cruz Biotechnology; anti-MTX2 (1:250) from Abnova and anti-FLAG (1:5000) from Sigma. Detection of the antibodies was performed with the chemiluminescent ECL Western Blotting System (Amersham).

2.8. Cell viability analysis

Stable FLPIn and knockdown cells to hNek5 were prepared for cell viability studies in 96-well plates (10×10^3 cells per well). The cells were induced for 48 h with 2 μg/ml tetracycline and, exposed to different drugs for 4 h at 37 °C. The hydrogen peroxide was administered at final concentration of 0, 10, 50, 150 and 300 μM; thapsigargin at 0, 10, 50, 150 and 300 μM. Cell viability was accessed by MTS assay, which was performed in triplicate. The measurement was performed using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the protocol provided. Formazan product is quantified by the amount of 490 nm absorbance, which is directly proportional to the number of living cells in culture. The data is normalized as a percentage of control.

2.9. Annexin V–FITC and propidium iodide staining

1×10^5 cells were stained with Annexin V–FITC and propidium iodide for 15 min at room temperature in the dark using Annexin V–FITC Apoptosis Detection Kit from BD Pharmingen™, followed by flow cytometry analysis (FACS). For statistical analysis, the double positive staining cells were considered.

2.10. Measurement of reactive oxygen species generation

General ROS production was measured using 2′, 7′-dichlorodihydrofluorescein (H2DCFDA), which is oxidized to a fluorescent product when exposed to ROS [16]. Cells were incubated with in standard reaction medium containing 5 μM of H2DCF-DA (Molecular Probes, Invitrogen, Carlsbad, CA) at 37 °C for 30 min before the thapsigargin treatment. Stable FLPIn and knockdown cells to hNek5 were treated with or without 2 μM thapsigargin for 4 h at 37 °C and 10 mM of pyruvate was used as a scavenger [17]. The cells were trypsinized and analyzed by a FACSCanto II flow cytometer (BD Biosciences). Results are given as mean fluorescence intensity (MFI). The analysis of ROS was performed in triplicate.

2.11. Statistical analysis

Data of measurement of ROS, apoptosis and viability are represented as the mean ± standard error of the mean (SEM). Data were analyzed using two-way ANOVA followed by Bonferroni post hoc tests using GraphPad Prism 4.0 software. A value of $p < 0.05$ was considered as statistically significant. All experiments were repeated at least three times.

2.12. Mitochondrial respiratory chain

Oxygen consumption was determined in a closed-chamber high-resolution respirometry OROBOROS Oxygraph-2K (OROBOROS Instruments, Innsbruck, Austria) equipped with a magnetic stirrer and temperature control set at 37 °C [18,19]. Approximately 2×10^6 viable cells stably expressing hNek5 or cells depleted for hNek5 were compared to FLAG control. The cells were suspended in KCl medium (65 mM KCl, 125 mM sucrose, 10 mM HEPES, 2 mM K_2HPO_4 and 1 mM $MgCl_2$, pH 7.2). Cells were permeabilized with 200 μM EGTA and 20 μM Digitonin maintaining intact mitochondrial membrane as previously described [20–22]. The steady-state basal respiratory flux was measured after permeabilization. The oxidative phosphorylation and mitochondrial respiratory activity were analyzed by the sequential addition of substrates and inhibitors of mitochondrial respiratory chain complexes in the following concentrations: 100 μM ADP, 2 μg/ml oligomycin, 100 nM FCCP, 2 μM rotenone, 5 mM succinate, 0.5 μM antimycin A and 200 μM TMPD/2 mM ascorbate. OROBOROS DatLab software was used to calculate the oxygen consumption.

3. Results

3.1. hNek5 is a partner of mitochondrial proteins

Nek5 has two major domains: an amino-terminal kinase domain and a regulatory C-terminal coiled-coil containing domain (C-terminus) (Fig. 1A). Among the members of the Nek family, the C-terminal shows great variability, and is involved in determining sub-cellular localization and to mediate interactions with other proteins [10]. Nek5 has also a Dead Box domain. This domain is involved in cellular processes such as pre-gene expression, mRNA processing and rearrangement of ribonucleoprotein complexes [23] (Fig. 1A).

We performed a yeast two-hybrid screen to identify hNek5 interacting partners, to learn about its interactome and function. The cDNA encoding the C-terminus of hNek5 (a.a. 260–708) was cloned in the pGBKT7 vector to be used as bait against the Mate & Plate Library — Universal Human (Normalized). By yeast two-hybrid screening among others, four positive clones were identified that encoded partial cDNA sequences of three proteins associated with mitochondrial functions: metaxin-2 (MTX2, represented by two cores of different size), BCL2-associated transcription factor 1 (BCLAF1) and cytochrome C oxidase assembly protein 11 (COX11) (Table 1, Fig. 1B). We found that the co-transfection of hNek5 C-terminus or its full length with MTX2 and BCLAF1 is capable to activate the reporters of interactions between these pairs of proteins in yeast. In contrast, the interaction with Cox11 was confirmed only for the C-terminal region (Fig. 1B).

For the interactions to occur *in vivo* they need to have approximate subcellular colocalizations. Accordingly, we studied their localization in mammalian cell lines (U2-OS) by confocal microscopy as shown in Fig. 1C, and found that they colocalize in both a diffuse but also a dotted fashion in the cell's cytoplasm, although Nek5 can also be detected additionally in the nucleus. The colocalization of Nek5 with COX11 and MTX2 showed a Pearson coefficient of 0.7609 and 0.6760, respectively. We did not obtain the Pearson coefficient for BCLAF1 because the image was not collected using the colocalization parameters.

The interactions between hNek5 and Cox11, MTX2 and BCLAF1 were also confirmed by immunoprecipitation using endogenous proteins or lysate of stable cells expressing hNek5. Hek293T or stably Nek5-Flag transfected cells lysates were subjected to co-immunoprecipitation using anti-Nek5 (Fig. 1D), anti-FLAG (Fig. 1E) or anti-BCLAF1 (Fig. 1F) antibody as the precipitating antibody, followed by Western blotting. All

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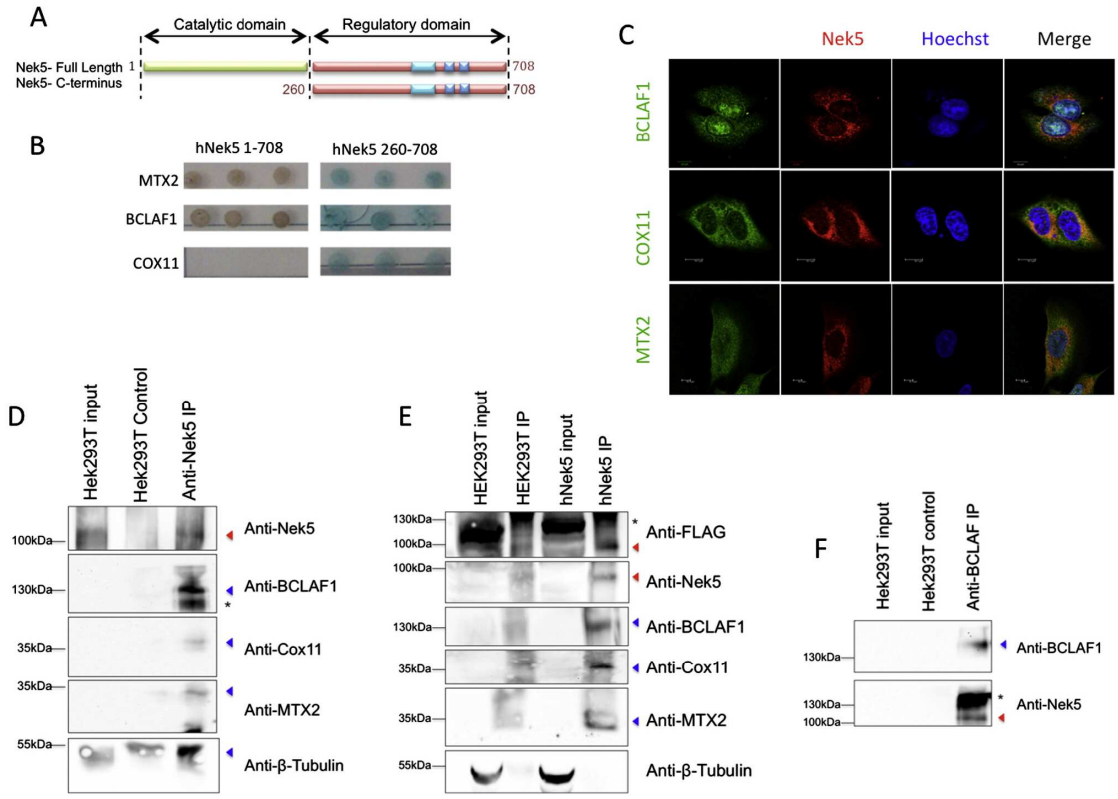

Fig. 1. hNek5 interacts with mitochondrial proteins. (A) Schematic representation of hNek5 functional domains and used constructs. The full length (a.a. 1–708) and the regulatory domain (a.a. 260–708) were cloned in plasmid pGBKT7 ('bait'). The two blue boxes represent the coiled-coil domain and the green box the dead-box domain. (B) Interaction by Y2H between hNek5(260–708) and MTX2, BCLAF1 and COX11. Protein–protein interactions were evaluated by the ability of the cells to grow on minimal medium in the presence of X-α-GAL. (C) Upper four rows: subcellular colocalization of endogenous hNek5 MTX2, BCLAF1 and COX11in U2-OS cells. Nuclei were stained using Hoechst dye. Merge is overlay of the hNek5, preys and Hoechst images. The Pearson coefficient was 0.7609 and 0.6760 for Cox11 and MTX2 respectively. Scale bars of 10 μm. (D) Immunoprecipitation of endogenous hNek5 of Hek293T cells with anti-Nek5 antibody and Western blot using anti-BCLAF1, anti-COX11, anti-MTX2 and anti-β-tubulin showed binding with Nek5 (lane 3). The control without antibody (Hek293T control) did not show any delectable binding (lane 2). (E) The hNek5 expression of HEK293-hNek5 TET-on cells was induced with tetracycline for 48 h. Immunoprecipitation of hNek5-FLAG with anti-FLAG resin and Western blot using anti-Nek5, anti-BCLAF1, anti-COX11, and anti-MTX2 but not anti-β-tubulin showed binding in Nek5 IP (lane 4). Hek293T cells were used as control and did not show any detectable binding (lane 2). (F) Hek293T cells were used to immunoprecipitate BCLAF1 using anti-BCLAF1 antibody and detection of co-precipitated Nek5 with anti-Nek5 (lane 3). (D, E and F) Red arrows represent the Nek5 expression; blue arrows are the proteins identified by Y2H screening and confirmed by immunoprecipitation. * represents unspecific staining or antibody chains. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

three targets were detected in the complex with hNek5 in the cell lysates of HEK293T or stable cells whereas no targets were found in complex with the negative control. The reverse co-immunoprecipitation was also confirmed by the detection of hNek5 after BCLAF1 immunoprecipitation. In conclusion, all three proteins (Cox11, MTX2 and BCLAF) are confirmed novel human Nek5 interactors.

3.2. hNek5 is a mitochondrial protein

As the location of hNek5 in cells is still unknown and some of its interactors have been reported as mitochondrial proteins or with functions related to this organelle [24–26], we analyzed if hNek5 is also localized to these cellular organelles. Therefore, we performed

Table 1
Human Nek5 interacting proteins identified by yeast two-hybrid system screens.

Protein interacting with Nek5[a]	Gene[b]	Uniprot accession number	Coded protein residues (retrieved/complete sequence)	Biological process	Potential phosphorylation sites[c]
Metaxin 2	MTX2	AAC25105.1	4–181 and 207–261 (263)	Cellular protein metabolic process, protein targeting to mitochondria	S98, S2, T221, T228, T141, S194, S10, T124, T224, S233
BCL2-associated transcription factor 1	BCLAF1	AAF64304.1	677–725 (920)	Induction of apoptosis, DNA-dependent, positive regulation of intrinsic apoptotic signaling pathway, positive regulation of response to DNA damage stimulus	T572, T564, S262, S609, S602, S154, S576, T472, S343, S359, S596, T664, S791, S196, T164, S237
Cytochrome c oxidase assembly protein 11	COX11	AAD08645.1	91–276 (276)	Respiratory chain complex IV assembly, respiratory gaseous exchange	S110, T81, S228, T26

[a] The proteins were selected from yeast cells under growth conditions in minimal medium without tryptophan, leucine, adenine and histidine and containing Aureobasidin and x-alfa-gal.
[b] Results from Blastx (GenBank).
[c] Sites predicted to be phosphorylated by Nek member using GPS 2.1.

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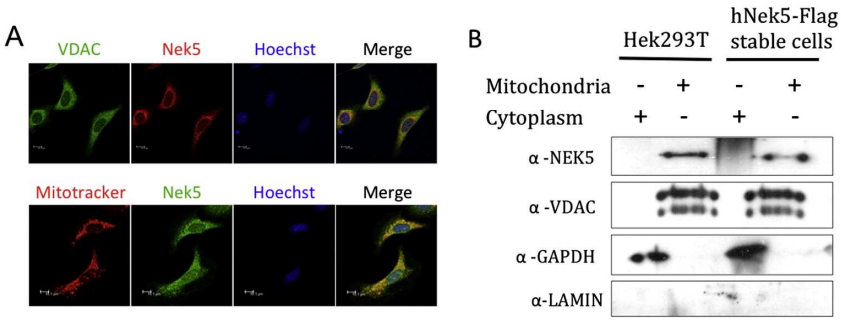

Fig. 2. Mitochondrial localization of hNek5. (A) Confocal fluorescence images of endogenous hNek5 (red or green) and VDAC (green), and Mitotracker Deep Red (red) in U2-OS cells. Left to right: hNek5, VDAC or Mitotracker Deep Red, Hoechst dye and merge. Merge is the overlay of the hNek5, VDAC or Mitotracker and Hoechst images. Mitochondria were visualized by antibody staining with the outer mitochondrial membrane markers VDAC (green) and Mitotracker Deep Red (red). Endogenous hNek5 (red when stained with VDAC and green when colocalized with MitoTracker Deep Red) detected with rabbit anti-Nek5 antibody colocalized with mitochondrial markers. Pearson coefficients for the shown images were 0.8397 and 0.7172 for VDAC and Mitotracker Deep Red respectively. (B) Western blot analysis of subcellular fractionation of HEK293 cells expressing hNek5 reveals presence of endogenous hNek5 proteins in the mitochondrial but not cytoplasmic fraction marked by VDAC and GAPDH respectively. Lamin A served as marker for the possible contaminating nuclear fraction. Scale bars of 10 μm. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

confocal microscopy analysis using mitochondrial marker (Mitotracker Deep Red and VDAC) and found that hNek5 colocalized with Mitotracker and VDAC in mitochondria of U2-OS cells with a Pearson coefficient of 0.7172 for Mitotracker Deep Red and 0.8397 for VDAC staining (Fig. 2A). The mitochondrial localization of Nek5 was also confirmed by cell fractionation and analysis of mitochondria content by Western blot. hNek5 co-migrated with the mitochondrial marker VDAC containing fraction and was not detected in the cytoplasmic fractions (Fig. 2B), characterized by GAPDH staining.

3.3. hNek5 reduces thapsigargin mediated cell death

As we confirmed the mitochondrial location of hNek5, it was relevant to access its role in this organelle. Beside the nuclear location, Bclaf1 interacts with Bcl2 family members and its overexpression was shown to result in apoptosis that was reversible in the presence of Bcl-2 or Bcl-xL [27]. To analyze the potential role of hNek5 in cell death regulation it was generated a Hek293 stable cell line that is tetracycline-inducible to express hNek5 protein (Fig. 1E, Anti-FLAG and Anti-Nek5 panels) and cells were challenged with hydrogen peroxide or thapsigargin and measured for cellular/mitochondrial viability (MTS assay). Nek5 stable expression showed an increased survival rate at high concentrations of hydrogen peroxide (150 and 300 μM), compared to cells that do not express Nek5 (Fig. 3A).

Thapsigargin inhibits Ca^{2+} ATPases and interferes thereby in intracellular Ca^{2+}levels, promoting collapse of the mitochondrial membrane potential, membrane fragmentation and loss of cell viability [28,29]. As MTS assay provides the measurement of mitochondrial enzymatic activity that reflects the number of viable cells, we performed it in order to address the effects of hNek5 on cell viability after thapsigargin treatment. After the treatment with different doses of thapsigargin for 4 h, the levels of cell viability had a slight increase in cells expressing hNek5 compared to HEK293T cells. The MTS results are consistent with apoptosis assay. After hNek5 induction for 48 h the cells were treated with thapsigargin (2 μM for 16 h) and the levels of cell death were accessed by Annexin V–FITC and propidium iodide assay. The presence of hNek5 resulted in decrease of cell death, suggesting an hNek5-mediated resistance to thapsigargin (Fig. 3B).

To study whether the decreased level of cell death is caused by endogenous formation of reactive oxygen species formation (ROS) we treated the stable cells with 2 μM thapsigargin for 4 h. Upon stable expression of hNek5 we observed a decrease of ROS formation (Fig. 3D). Furthermore, pyruvate was used as ROS scavenger, since it has been show to be taken up actively by cells via monocarboxylate transporters and to act as a potent intracellular scavenger for H_2O_2 [30–33]. hNek5 expression was shown to act in synergism with pyruvate and reduce further the levels of ROS in the cells (Fig. 3C). Based on that, hNek5 expression decreases the effects of thapsigargin on cell death through the direct or indirect reduction of formation of reactive oxygen species and may thereby act as an anti cell death protein.

3.4. hNek5 depletion increases cell death

Since hNek5 expression seemed to exhibit protective effects in the context of cell death regulation, we asked whether hNek5-deficient cells would show deficiencies in their ability to promote cell death. shRNA lentiviral particles were transduced in Hek293T cells allowing the integration of the shRNA constructs into the genomic DNA of target cells.

The silencing resulted in an over 60% decrease in the relative mRNA levels (normalized with tubulin m-RNA levels) of hNek5 (Fig. 4A), as assessed by RT-PCR. Cells depleted for Nek5 were then treated with hydrogen peroxide for 4 h and the cell viability assessed by MTS. Indeed, as expected, cells depleted for hNek5 displayed a significant decrease in cell viability (Fig. 4B and data not shown) upon challenge with H_2O_2.

Next, after 16 h of treatment with thapsigargin, Annexin V–FITC and propidium iodide staining was used to access the levels of cell death. In contrast to tetracycline-inducible hNek5 cells, the number of FITC and PI positive cells was higher in cells depleted of hNek5 compared to HEK293T cells (Fig. 4C). After 4 h of thapsigargin treatment, we observed a higher ROS formation in hNek5-deficient cells (Fig. 4D) but not in the apoptotic levels, suggesting that the increase on ROS levels precedes cell death. Together, these data confirm the effects of Nek5 in the cell death process, acting as a possible anti-apoptotic protein.

3.5. The rate of mitochondrial respiration increases in cells depleted for hNek5

Our findings suggest an increase in ROS production in cells depleted for hNek5 and treated with thapsigargin. This may suggest a possible regulatory, activity of hNek5 towards, mitochondrial functions. Therefore we examined in more detail the effects of hNek5 on mitochondrial respiration, using permeabilized cells.

Mitochondrial respiratory chain activity of stable and depleted hNek5 cells was determined using the *Oroboros Oxygraph* in the presence of substrates and inhibitors of the mitochondrial respiratory chain. Fig. 5A demonstrates typical measurements of oxygen

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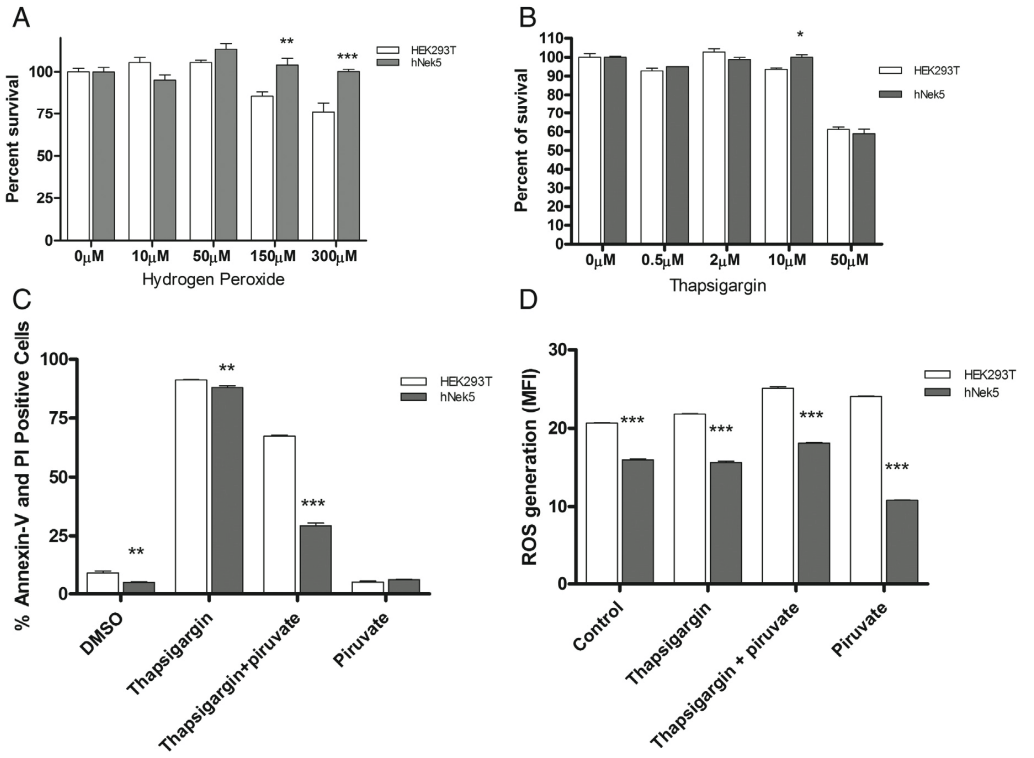

Fig. 3. hNek5 is involved in cell survival after thapsigargin treatment. (A) Hydrogen peroxide (10, 50, 150 and 300 μM) or (B) thapsigargin (1, 10, 50,100 and 330 μM) treatment for 4 h. Cell viability was measured by MTS. Analysis of viability levels for the treatments was made by two way ANOVA and Bonferroni post test. * = $0.05 < p < 0.01$; ** = $0.01 < p < 0.001$; *** = $p < 0.001$. (C) Hek93T-hNek5 cells were treated with thapsigargin for 16 h. Apoptotic cells were detected by Annexin V and propidium iodide double staining followed by flow cytometry analysis. (D) The intracellular ROS concentration was measured using the DCFDA assay. The median peak determined the intensity of staining. The data are presented as the mean ± SD. Statistical analysis was made by two-way ANOVA and Bonferroni post-test was used. * = $0.05 < p < 0.01$; ** = $0,01 < p < 0.001$; *** = $p < 0.001$.

consumption in cells. Basal respiration was measured in the presence of EGTA, site I substrates and digitonin. State 3 coupled respiration was induced by the addition of ADP. After this measurement, the specific ATP-synthase inhibitor, oligomycin, was added to inhibit oxidative phosphorylation, and then the uncoupling was induced by addition of the protonophore FCCP. Next, rotenone was used as a Complex I inhibitor to block the transfer of electrons from iron-sulfur centers in complex I to ubiquinone. Subsequently, succinate was applied to certify the operation of complex II and then uncoupled respiration was inhibited at complex III by addition of antimycin A. Ascorbate (Asc) and *N*,*N*,*N*,*N*-tetramethyl-*p*-phenylenediamine (TMPD) were then added to access the activity of cytochrome c oxidase (complex IV). TMPD is an artificial redox mediator that participates in the transfer of electrons from ascorbate to cytochrome c (Fig. 5A).

Defects through the respiratory chain were more pronounced during basal respiration, and after succinate or ascorbate/TMPD addition in hNek5 stable and depleted cells. The O2 flux in basal conditions was 1.7 fold higher in shRNA-hNek5 cells (Fig. 5B) indicating that silenced cells had increased respiratory activity. The succinate and TMPD/Ascorbate addition, substrates for complex II and IV respectively, resulted in higher rates of oxygen consumption in the hNek5 silenced cells (Fig. 5C and D). The expression of Nek5 on the other hand caused an inhibition of mitochondrial respiration, also with most prominent effects at the complexes II and IV (Fig. 4B–D). hNek5 cells resulted in approximately 16% reduction in respiratory rates after succinate addition whereas the addition of TMPD/ascorbate decreased the respiratory activity in 22% compared to HEK293T cells (Fig. 5C and D).

4. Discussion

hNek5 belongs to the NIMA-related kinase (Nek) family but is among the least studied members of this family. Shimizu et al. (2013) identified hNek5 as a key substrate of caspase-3, promoting skeletal muscle differentiation. The levels of hNek5 increase during differentiation and the expression of hNek5 was able to exert both promyogenic and pro-apoptotic effects [13].

A yeast two-hybrid assay using the C-terminus of hNek5 as 'bait' allowed us to identify MTX2, BCLAF1 and COX11 as hNek5 partners. MTX2 is located on the cytosolic face of the mitochondrial outer membrane and interacts with metaxin-1. This complex plays a role in the protein import into mammalian mitochondria [34]. Cytochrome c oxidase (Cox) is the terminal member of the respiratory chain, and promotes reduction of oxygen to water. Complex IV also translocates protons from the matrix to the intermembrane space, across the inner mitochondrial membrane [35]. The assembly of functional complex IV, is a multistep process and accessory proteins are necessary for the correct translation, post-translational modification, processing, membrane insertion, assembly of the structural subunits, as well as, the synthesis and insertion of the prosthetic groups [36–38].

In the Cox structure the two copper ions centers are designated as Cu_A and Cu_B that are coordinated within Cox2 and Cox1, respectively [39]. The Cu_B site formation is dependent of cytochrome c oxidase assembly protein 11 (Cox11). It was demonstrated in Δ*cox11* cells that the cytochrome c oxidase isolated from *R. sphaeroides* lacked Cu_B, but contained both hemes [40]. Nek5's interactor, Cox11, is anchored in

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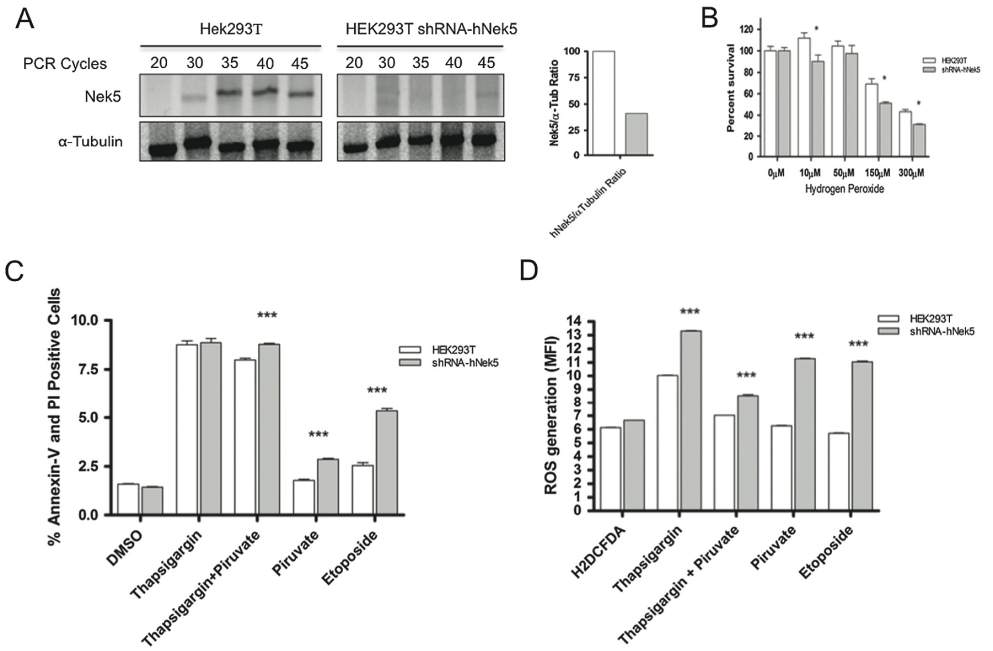

Fig. 4. The knockdown of hNek5 potentializes the effects of thapsigargin on cell death. (A) HEK293T cells were silenced for hNek5 (shNek5) and the expression of mRNA was analyzed by RT-PCR using specific primers for hNek5 amplification. The levels of hNek5 mRNA were measured by band intensity and normalized by α-tubulin. (B) 10×10^3 of shNek5 cells were exposed to hydrogen peroxide (10, 50, 150 and 300 μM) treatment for 4 h. Cell viability was measured by MTS assay. (C and D) Cells were exposed to thapsigargin (2 μM) and etoposide (30 μg/ml) treatment for 4 h. (C) Annexin V and propidium iodide double staining were used to access apoptotic cells. (D) The intracellular ROS concentration was measured using the DCFDA assay. All the experiments were performed in triplicate. Analysis of viability levels for the treatments was made by two way ANOVA and Bonferroni post test. * = $0.05 < p < 0.01$; ** = $0,01 < p < 0.001$; *** = $p < 0.001$. The data are presented as the mean ± SD.

the inner mitochondrial membrane and exerts protein chaperone roles in folding and stabilizing both Cox1 and Cox2 chains [41].

Finally, the hNek5 interacting protein, BCALF1 is a nuclear protein identified in a yeast two-hybrid screen against the adenoviral Bcl-2 homologue E1B19K and was shown to interact with apoptosis regulators Bcl-2 and Bcl-x_L in humans [42]. Aside being a nuclear protein, BCLAF1 exerts also important roles in the cytoplasm and mitochondria. BCLAF1 has a substantial role in activation of caspase-dependent mitochondrial-mediated apoptotic pathways in cells responding ionizing radiation (IR)-responsive cells [26].

On identifying these mitochondria associated hNek5 interactors, we next tested if hNek5 is localized to mitochondria. Both confocal microscopy and cell fractioning followed by Western blot showed the mitochondrial localization of hNek5. The Nek family members are usually involved in one or more than one of the three Neks core functions: 1. centrioles/mitosis, 2. primary ciliary function/ciliopathies and 3. DNA damage response [43]. To execute these functions they are generally localized in the nucleus or cytoplasm (centrioles, cilium, etc.). However, besides the nuclear and cytoplasmic functions, Nek1 was the first member of Nek family related to mitochondria. In mitochondria, Nek1 is involved in cell death regulation through phosphorylation of VDAC1, regulating its opening and closing [12,44].

Thapsigargin treatment was used in this approach after a screening of different drugs involved in cell death (data not shown). Thapsigargin is a well-known inhibitor of Ca^{2+}-ATP-ases, both in sarcoplasmic and endoplasmic reticulum [45]. This drug can also increase calcium levels, promote mitochondrial depolarization and fragmentation, and cell viability loss [29].

After thapsigargin treatment, the stable expression of hNek5 results in a decrease of cell death, suggesting an hNek5-mediated susceptibility to thapsigargin. Furthermore, we observed a decrease of ROS formation upon stable Nek expression. In agreement with these findings the number of dead cells was higher in cells depleted for hNek5 and ROS formation rates were higher.

In the same line, the presence or higher expression of hNek5 decreases the effects of thapsigargin on cell death through the reduction of ROS formation and suggests that Nek5 may act as an anti cell death protein. Nek1 acts also as an anti-cell death protein [12]. It phosphorylates VDAC1 on Ser193 to prevent excessive cell death after injury, closing VDAC and preventing cytochrome c efflux [44]. In addition to hNek1 and hNek5, hNek2 depleted cells also overcame cancer cell drug resistance, and entered in apoptosis *in vitro* in a xenograft myeloma mouse model [46].

Our report did not show the mechanism of action of hNek5 on cell death. But based on yeast two-hybrid screening is possible to assume that the mechanism may occur through the identified interacting proteins, or other mitochondrial related effects. The hNek5's interactors are all associated with cell death regulation events. MTX2-depleted mitochondria were deficient in import and assembly of VDAC and Tom40 [47]. Metaxin-1 and 2 are required for TNFα-induced cell death [48,49]. Mitochondrial Bak is part of VDAC2/Mtx1/Mtx2 multi-protein complex and after the induction of apoptosis, Bak switches from its association with Mtx2 and VDAC2 to interact with Mtx1 [24]. BCLAF1, another hNek5 interactor, may negatively regulate the DNA repair process mediated by Ku70/p18-cyclin E complex, promoting apoptosis [50]. In irradiated cells, BCLAF1 promoted apoptosis through disturbing p21-mediated inhibition of caspase/cyclin E-dependent, mitochondrial-mediated pathway [26].

Isolated defects of COX activity have been described in numerous mitochondrial disorders [51]. Studies of human/yeast Cox11 chimeric molecules revealed the replacing the yeast Cu(I)-binding domain by the human equivalent failed to generate a functional complex. Sequence differences in the human protein may attenuate an interaction for a Cu(I) donor protein or a Cox1-associated protein. The inability of

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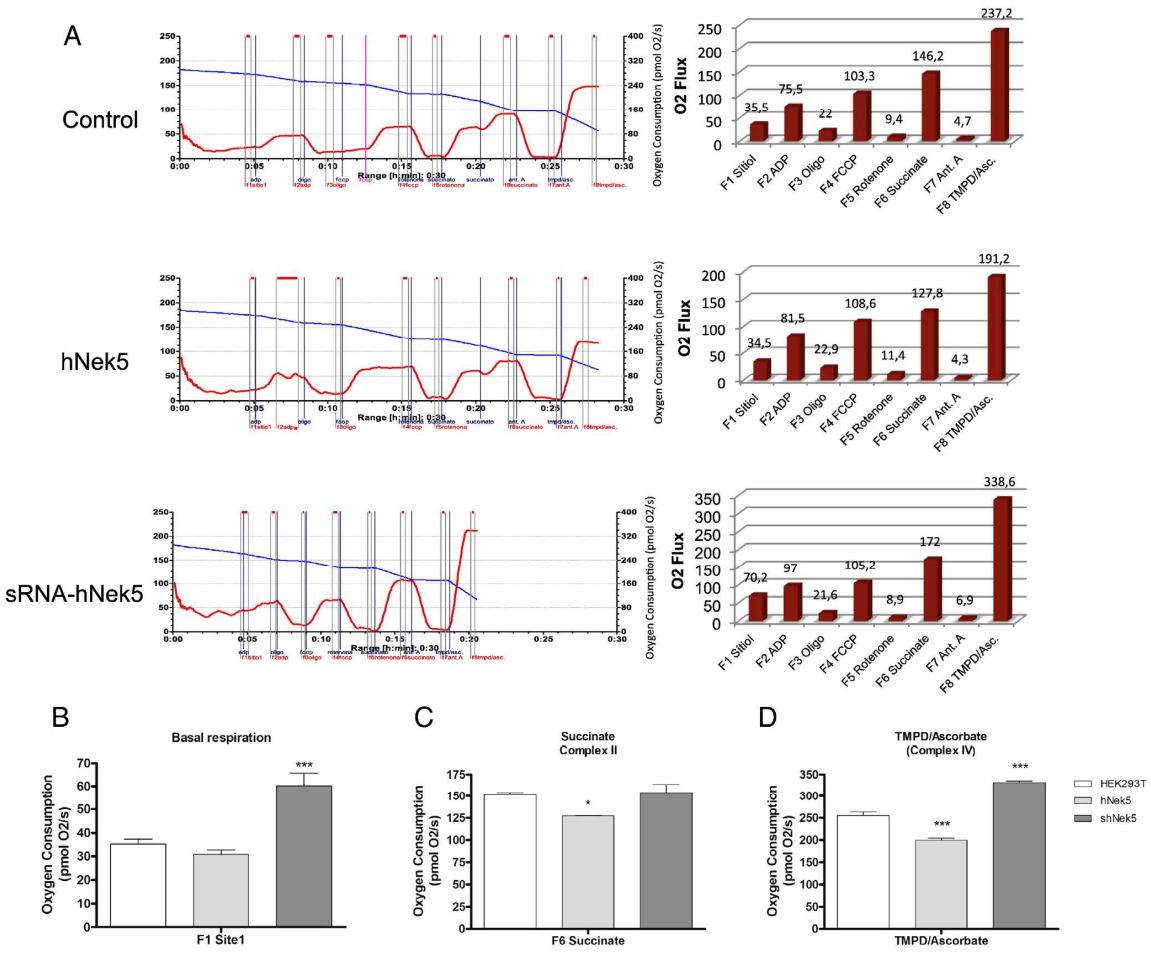

Fig. 5. Mitochondrial respiration had the activity reduced by stable expression of hNek5. (A) Representative profiles of oxygen consumption in permeabilized cells. Oxygen consumption was assessed in HEK293T, hNek5 and shRNA-hNek5 cells using oxygraphy as described under Materials and methods. ADP, adenosine diphosphate; oligo, oligomycin; FCCP, anti. A, antimycin A; Asc, ascorbate; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride. Basal respiration (B), succinate (C) and the respiration rate with Asc/TMPD (complex IV) (D) were measured in HEK293T, stable hNek5 expressing cells and shRNA-hNek5 knocked down cells. Values are means SD, $n = 3$. Analysis of oxygen consumption comparing cells in the presence or not of hNek5 was made by two way ANOVA and post-test was used to compare oxygen consumption between cells. * = $0.05 < p < 0.01$; ** = $0,01 < p < 0.001$; *** = $p < 0.001$.

human *COX11* to complement yeast Δ*cox11* cells suggests that human Cox11 may be impaired in its interaction with another protein, perhaps Cox1 itself or could be regulated by post-translational modification as phosphorylation.

Steenaart and Shore (1997) found that COX was phosphorylated on subunit IV-1 by labeling mitochondrial proteins with radioactive ATP [51]. Since then other phosphorylation sites have been mapped on COX [52–60]. *In silico* analysis of human Cox11 sequence (GPS 2.0 software, CUCKOO Workgroup) suggests the phosphorylation of serines 110 and 228 or the threonine 26 and 81 by members of the Nek family [61] (Table 1). The interaction between hNek5 and Cox11 was also confirmed by immunoprecipitation but phosphosites were not analyzed at this time.

In yeast, *Cox11* mutants, but not Δ*cox4 and* Δ*cox17* display hypersensitivity to hydrogen peroxide treatment, suggesting a possible secondary role for Cox11 in oxidative stress responses [62-63]. The peroxide hypersensitivity is probably linked to the functionality of the Cox11 molecule. Mutants with a partial respiratory deficiency proved insensitive to peroxide suggesting that even partially functional Cox11 can fully protect against peroxide exposure [62]. The selective peroxide sensitivity of Δ*cox11* cells suggests that Cox11 has a mitochondrial function independent of copper metal coordination and Cox folding.

Mitochondria are important not only for providing energy through ATP synthesis in the electron transport chain, but also have a central role in the activation of cell death pathways. The inhibition of the electron transport chain leads to failures in energy generation, thereby increasing the cells vulnerability to apoptosis [64].

Metabolic alterations in Alzheimer's disease involve the impairment of the mitochondrial electron transport chain, specially, cytochrome c oxidase (COX) activity [65]. Thereby, ER-to-mitochondria Ca^{2+} transfer plays an essential role during Aβ-induced neuronal apoptosis [66, 67]. Mitochondrial impairment due to COX inhibition increases the susceptibility to Aβ-induced ER stress-mediated apoptosis or thapsigargin treatment, pointing to the important crosstalk between ER and mitochondria during Aβ-induced neuronal cell death in Alzheimer's disease [68].

Four hours after treatment with thapsigargin, the depletion of hNek5 resulted in an increase of the ROS production and 16 h

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after the treatment the cells entered in apoptosis (Fig. 4). The mitochondrial electron transport chain is a potential source for the production of reactive oxygen species (ROS) [69]. In yeast the reduction of COX complex function correlates with increased ROS levels in vivo [70].

Based on the foregoing, the exact mechanism of action of Nek5 in the cell death process was not fully elucidated, however, this work has opened several perspectives to be explored in the future as the possible involvement of Nek5 in cell death and mitochondrial dysfunction through COX11, MTX2 and BCLAF1. Nek5 represents a predictor for drug resistance and could be an important target for cancer therapy.

5. Conclusions

In conclusion our data suggest for the first time the hNek5 mitochondrial localization and its role on cell death and cell respiration defects. Stable expression of h*Nek5* in Hek293T cells resulted in enhanced cell viability, decrease on cell death, and drug resistance, while targeting h*Nek5* knockdown by shRNA, on the other hand, overcame cancer cell drug resistance and induced apoptosis *in vitro*.

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References

[1] P.A. Kaliman, V.V. Lemeshko, V.V. Petrenko, Ukr. Biokhim. Zh. 53 (1) (1981) 35–38.
[2] J.P. Silva, M. Köhler, C. Graff, A. Oldfors, M.A. Magnuson, P.O. Berggren, N.G. Larsson, Nat. Genet. 26 (3) (2000) 336–340 (PMID: 11062475).
[3] H.M. McBride, M. Neuspiel, S. Wasiak, Curr. Biol. 16 (14) (2006) R551–R560 [(PMID: 16860735).
[4] S.A. Susin, H.K. Lorenzo, N. Zamzami, I. Marzo, B.E. Snow, G.M. Brothers, J. Mangion, E. Jacotot, P. Costantini, M. Loeffler, N. Larochette, D.R. Goodlett, R. Aebersold, D.P. Siderovski, J.M. Penninger, G. Kroemer, Nature 397 (1999) 441–446 (PMID: 9989411).
[5] A. Gross, J.M. McDonnell, S.J. Korsmeyer, Genes Dev. 13 (1999) 1899–1911 (PMID: 10444588).
[6] D.R. Winge, Mol. Cell. Biol. 32 (14) (2012) 2647 (PMID: 22586278).
[7] B.R. Oakley, et al., J. Cell Biol. 4 (1983) 1155–1158 (PMID: 6339527).
[8] S.A. Osmani, G.S. May, N.R. Morris, J. Cell Biol. 104 (1987) 1495–1504 (PMID: 3294854).
[9] S.A. Osmani, R.T. Pu, N.R. Morris, Cell 53 (1988) 237–244 (PMID: 3359487).
[10] M.J. O'Connell, M.J. Krien, T. Hunter, Trends Cell Biol. 13 (5) (2003) 221–228 (PMID: 12742165).
[11] G.V. Meirelles, A.M. Perez, E.E. de Souza, F.L. Basei, P.F. Papa, T.D. Melo Hanchuk, V.B. Cardoso, J. Kobarg, World J. Biol. Chem. 5 (2) (2014) 141–160.
[12] Y. Chen, W.J. Craigen, D.J. Riley, Cell Cycle 15; 8 (2) (2009) 257–267 (PMID: 19158487).
[13] K. Shimizu, T. Sawasaki, FEBS Lett. 587 (14) (2013) 2219–2225 (PMID: 23727203).
[14] M.F. Carazzolle, L.M. De Carvalho, H.H. Slepicka, R.O. Vidal, G.A. Pereira, J. Kobarg, G.V. Meirelles. PLoS ONE 9 (6) (2014) e100385 (PMID: 24949626).
[15] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254 (PMID: 942051).
[16] C.L. Murrant, M.B. Reid, Microsc. Res. Tech. 55 (2001) 236–248 (PMID: 11748862)].
[17] X. Wang, E. Perez, R. Liu, L.J. Yan, R.T. Mallet, S.H. Yang, Brain Res. 1132 (1) (2007) 1–9 (Feb 9, 2007. [PMID: 17174285).
[18] F.A. Rossato, K.G. Zecchin, P.G. La Guardia, R.M. Ortega, L.C. Alberici, R.A.P. Costa, R.R. Catharino, E. Graner, R.F. Castilho, A.E. Vercesi, PLoS ONE 9 (6) (2014) e101060 (PMID: 24964211).
[19] P. Sirvent, S. Bordenave, M. Vermaelen, B. Roels, G. Vassort, et al., Biochem. Biophys. Res. Commun. 338 (2005) 1426–1434 (PMID: 16271704).
[20] C. Malaguti, P.G. La Guardia, A.C. Leite, D.N. Oliveira, R.L. de Lima Zollner, R.R. Catharino, A.E. Vercesi, H.C. Oliveira, Free Radic. Res. 48 (12) (2014) 1494–1504 (PMID: 25236567).
[21] A.E. Vercesi, C.O. Rodrigues, S.A. Uyemura, L. Zhong, S.N. Moreno, J. Biol. Chem. 273 (1998) 31040–31047 (PMID: 9813002).
[22] S.K. Chowdhury, Z. Drahota, D. Floryk, P. Calda, J. Houstek, Clin. Chim. Acta 298 (2000) 157–173 (PMID: 10876012).
[23] C.L. Young, S. Khoshnevis, K. Karbstein, Proc. Natl. Acad. Sci. U. S. A. 110 (29) (2013) E2668–E2676 (PMID: 23630256).
[24] P.F. Cartron, E. Petit, G. Bellot, L. Oliver, F.M. Vallette, Cell. Signal. 26 (2014) (2014) 1928–1934 (PMID: 24794530).
[25] A.I. Tzagoloff, M. Nobrega, N. Gorman, P. Sinclair, Biochem. Mol. Biol. Int. 31 (3) (1993) 593–598 (PMID: 8118433).
[26] Y.Y. Lee, Y.B. Yu, H.P. Gunawardena, L. Xie, X. Chen, Cell Death Dis. 26 (3) (2012) e359 [PMID: 22833098].
[27] G.M. Kasof, L. Goyal, E. White, Mol. Cell. Biol. 19 (1999) 4390–4404 (PMID: 10330179).
[28] A.E. Vercesi, S.N. Moreno, C.F. Bernardes, A.R. Meinicke, E.C. Fernandes, R. Docampo, J. Biol. Chem. 268 (12) (1993) 8564–8568 (PMID: 8473301).
[29] R.A. Quintanilla, Y.N. Jin, R.V. Bernhardi, G.V.W. Johnson, Mol. Neurodegener. 8 (2013) 45 (PMID: 24330821).
[30] R. Franco, M.I. Panayiotidis, J.A. Cidlowski, J. Biol. Chem. 282 (42) (2007) 30452–30465 (PMID: 17724027).
[31] T. Shimizu, T. Numata, Y. Okada, Proc. Natl. Acad. Sci. U. S. A. 27;101 (17) (2004) 6770–6773 (PMID: 15096609).
[32] R. Reinehr, S. Becker, A. Eberle, S. Grether-Beck, D. Haussinger, J. Biol. Chem. 280 (29) (2005) 27179–27194 (PMID: 15917250).
[33] C.D. Bortner, J.A. Cidlowski, J. Biol. Chem. 278 (40) (2003) 39176–39184 (PMID: 12821680).
[34] L.C. Armstrong, A.J. Saenz, P. Bornstein, J. Cell. Biochem. 74 (1999) 11–22 (PMID: 10381257).
[35] T.H. Tsukihara, E. Aoyama, T. Yamashita, H. Tomizaki, K. Yamaguchi, R. Shinzawa-Itoh, R. Nakashima, Yaono, S. Yoshikawa, Science 272 (1996) 1136–1144 (PMID: 8638158).
[36] J.E. McEwen, C. Ko, B. Kloeckner-Gruissem, R.O. Poyton, J. Biol. Chem. 261 (1986) 11872–11879 (PMID: 3017950).
[37] A. Tzagoloff, N. Capitanio, M.P. Nobrega, D. Gatti, EMBO J. 9 (1990) 2759–2764 (PMID: 2167832).
[38] H.S. Carr, G.N. George, D.R. Winge, J. Biol. Chem. 277 (2002) 31237–31242 (PMID: 12063264).
[39] H.S. Carr, A.B. Maxfield, Y.C. Horng, D.R. Winge, Biol. Chem. 280 (2005) 22664–22669 (PMID: 15840584).
[40] L. Hiser, M. Di Valentin, A.G. Hamer, J.P. Hosler, J. Biol. Chem. 275 (2000) 619–623 (PMID: 10617659).
[41] O. Khalimonchuk, K. Ostermann, G. Rodel, Curr. Genet. 47 (2005) 223–233 (PMID: 15776235).
[42] G.M. Kasof, L. Goyal, E. White, Mol. Cell. Biol. 19 (6) (1999) 4390–4404 (PMID: 10330179).
[43] Y. Chen, M. Gaczynska, P. Osmulski, R. Polci, D.J. Riley, Biochem. Biophys. Res. Commun. 394 (3) (2010) 798–803 (PMID: 2023078).
[44] P. Korge, J.N. Weiss, Eur. J. Biochem. 265 (1999) (273 ± 280 (1999) q FEBS 1999. [PMID: 10491183).
[45] Y. Zhou, L. Wen, J. Ren, Protein Eng. Des. Sel. 24 (3) (2011) 255–260 (PMID: 21062758).
[46] Kozjak-Pavlovic, V; Ross, K; Benlasfer, N; Kimmig, S; Karlas, A; Rudel, T. (2007). Conserved roles of Sam50 and metaxins in VDAC biogenesis. EMBO Rep. 2007 Jun;8(6):576–82. [PMID: 17510655].
[47] K.M. Abdul, K. Terada, M. Yano, M.T. Ryan, I. Streimann, N.J. Hoogenraad, M. Mori, Biochem. Biophys. Res. Commun. 276 (2000) 1028–1034 (PMID: 11027586).
[48] X. Wang, K. Ono, S.O. Kim, V. Kravchenko, S.C. Lin, J. Han, EMBO Rep. 2 (2001) 628–633 (PMID: 11454742).
[49] S. Mazumder, D. Plesca, A. Almasan, Cell Cycle 6 (2007) 1437–1442 (PMID: 17581275).
[50] M. Zeviani, C.T. Moraes, S. DiMauro, H. Nakase, E. Bonilla, E.A. Schon, L.P. Rowland, Neurology 51 (6) (1998) 1525–1533 (PMID: 3412580).
[51] N.A. Steenaart, G.C. Shore, FEBS Lett. 415 (1997) 294–298 (PMDI: 9357986).
[52] T. Tsukihara, K. Shimokata, Y. Katayama, H. Shimada, K. Muramoto, H. Aoyama, M. Mochizuki, K. Shinzawa-Itoh, E. Yamashita, M. Yao, Y. Ishimura, S. Yoshikawa, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 15304–15309 (PMDI: 14673090).
[53] I. Lee, A.R. Salomon, S. Ficarro, I. Mathes, F. Lottspeich, L.I. Grossman, M. Hüttemann, J. Biol. Chem. 280 (2005) 6094–6100 (PMDI: 15557277).
[54] I. Lee, A.R. Salomon, K. Yu, J.W. Doan, L.I. Grossman, M. Hüttemann, Biochemistry 45 (2006) 9121–9128 (PMDI: 16866357).
[55] J.K. Fang, S.K. Prabu, N.B. Sepuri, H. Raza, H.K. Anandatheerthavarada, D. Galati, J. Spear, N.G. Avadhani, FEBS Lett. 581 (2007) 1302–1310 (PMDI: 17349628).
[56] S. Helling, S. Vogt, A. Rhiel, R. Ramzan, L. Wen, K. Marcus, B. Kadenbach, Mol. Cell. Proteomics 7 (2008) 1714–1724 (PMDI: 18541608).
[57] L. Samavati, I. Lee, I. Mathes, F. Lottspeich, M. Hüttemann, J. Biol. Chem. 283 (2008) 21134–21144 (PMDI: 18534980).
[58] J.V. Olsen, M. Vermeulen, A. Santamaria, C. Kumar, M.L. Miller, L.J. Jensen, F. Gnad, J. Cox, T.S. Jensen, E.A. Nigg, S. Brunak, M. Mann, Sci. Signal. 3 (104) (2010) ra3 (PMID: 20068231).
[59] M. Hüttemann, S. Helling, T.H. Sanderson, C. Sinkler, L. Samavati, G. Mahapatra, A. Varughese, G. Lu, J. Liu, R. Ramzan, S. Vogt, L.I. Grossman, J.W. Doan, K. Marcus, I. Lee, Biochim. Biophys. Acta 17 (4) (2011) 598–609 (PMID: 21771582).
[60] Y.C. Homg, P.A. Cobine, A.B. Maxfield, H.S. Carr, D.R. Winge, J. Biol. Chem. 279 (2004) 35334–35340 (PMID: 15199057).
[61] Y. Xue, Z. Liu, J. Cao, Q. Ma, X. Gao, Q. Wang, C. Jin, Y. Zhou, L. Wen, J. Ren, Protein Engineering, Design and Selection 24 (3) (2010) 255–260.
[62] G.S. Banting, D.M. Glerum, Cell 5 (2006) 568–578 (PMID: 16524911).
[63] C. Pungartnik, M.F. Kern, M. Brendel, J.A.P. Henriques, Curr. Genet. 36 (124–129) (1999) 16 (PMID: 10501934).
[64] J.Q. Kwong, M.S. Henning, A.A. Starkov, G. Manfredi, J. Cell Biol. 179 (2007) 1163–1177 (PMID: 18086914).
[65] A.M. Pickrell, H. Fukui, C.T. Moraes, J. Bioenerg. Biomembr. 41 (2009) 453–456 (PMID: 19795195).

[66] R.O. Costa, E. Ferreiro, S.M. Cardoso, C. Oliveira, C.M. Pereira, J. Alzheimers Dis. 20 (2010) 625–636 (PMID: 20182029).
[67] R.O. Costa, E. Ferreiro, I. Martins, I. Santana, S.M. Cardoso, C.R. Oliveira, C.M. Pereira, Neurobiol. Aging 33 (824) (2012) (e825-816. [PMID: 21704433).
[68] R.O. Costa, E. Ferreiro, C.R. Oliveira, C.M.F. Pereira, Mol. Cell. Neurosci. 52 (2012) 1–8 (PMID: 23026561).
[69] T.R. Figueira, M.H. Barros, A.A. Camargo, R.F. Castilho, J.C. Ferreira, A.J. Kowaltowski, F.E. Sluse, N.C. Souza-Pinto, A.E. Vercesi, Antioxid. Redox Signal. 18 (16) (Jun 1 2013) 2029–2074. http://dx.doi.org/10.1089/ars.2012.4729 (PMID: 23244576).
[70] J.E. Leadsham, G. Sandes, S. Giannaki, E.L. Bastow, R. Hutton, W.R. Naeimi, Breitenbach, C.W. Mand Gourlay, Cell Metab. 18 (2013) 279–286 (PMID: 23931758).

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Artigo III

The activity of TTLL4 in the polyglutamylation process is reduced by Nek5 phosphorylation

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(Artigo em preparação)

The activity of TTLL4 in the polyglutamylation process is reduced by Nek5 phosphorylation

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Author contributions: TDMH, MMM, AW, CJ and JK performed the literature search, data analysis and contributed specific parts of the manuscript. TDMH performed the experiments with help from MMM and AW. TDMH and JK are elaborating the final version of the text and JK supervised the project.

INTRODUCTION

The microtubule cytoskeleton is essential for the internal organization of eukaryotic cells and are involved in cell division, differentiation and neuronal transport inside the cells (Bieling et al., 2008). The great diversity of tubulin inside the cells is due to the expression of tubulin isotypes and a large number of post-translational modifications (PTM) as acetylation / deacetylation, removal and addition of tyrosine in the C-terminal region, phosphorylation, and polyglycilation and polyglutamylation (Eddé et al., 1990; Redeker et al., 1994).

The glutamylation was initially discovered in tubulin and consists of the addition of glutamate side chains to proteins (Eddé et al., 1990; Redeker et al., 1994). It is found in microtubule cilia and flagella (Lechtreck & Geimer, 2000), centrioles, basal bodies and centrosomes (Bobinnec et al., 1998b). During the division, mitotic spindle microtubules have increased levels of glutamylated tubulin (Regnard et al., 1999; Verhey & Gaertig, 2007).

In vitro experiments have shown that the state of polyglutamylation in α or β tubulin microtubules act as regulator of tubulin interaction with MAPs and kinesins (Boucher et al., 1994; Larcher et al., 1996; Bonnet et al., 2001). The *tubulin tyrosine ligase* (TTL) was the first enzymes involved in modification of tubulin (Ersfeld et al., 1993). Immunoprecipitation assays of microtubule neurons allowed the purification of tubulin complexed with *tubulin tyrosine ligase like 1* (TTLL1) protein (Janke et al., 2005). Studies of the catalytic activity of TTLL family members revealed that the heterogeneity of microtubules poly-modifications is due to specificity of substrates of these enzymes. But polyglutamylation is not restricted to tubulins. It has been shown that occur in nucleosome assembly protein NAP1 NAP2 (Regnard et al., 2000), and probably in many proteins not yet been identified. Glutamine rich proteins may have also a “natural role” in deregulated inducing cell death in neurodegenerative diseases (Blum et al., 2012). TTLL4 is a protein of TTLL family and is capable to catalyze ligations of diverse amino acids like tyrosination, polyglycylation, and polyglutamylation (Westermann et al., 2005). Besides to catalyse the tubulins polyglutamylation, TTLL4 and TTLL5 were shown to have the ability to polyglutamylated nontubulin proteins (van Dijk et al., 2008).

Although Neks comprise a family of proteins that regulate mitotic events including entering mitosis, centrosomes separation, spindle formation, and cytokinesis (O'Connell et al., 2003; O'Regan et al., 2007), Westermann et al., (2003) detected the first evidence in *Crithidia fasciculata* of the involvement of Nek in polyglutamylation. Chromatography experiments using hydroxyapatite as a final step copurified polyglutamilated tubulins with CfNek. In addition, purified extract of CfNek5 was capable to polyglutamylated tubulins *in vitro*.

The NIMA-related kinases (Neks) are mammalian enzymes with high identity (40-45%) to the Aspergillus nidulans mitotic protein NIMA (never in mitosis gene A) within their catalytic domain (O'Regan et a., 2007; Belham et al., 2003). NIMA is related to mitotic entry, chromatin condensation, spindle organization, and cytokinesis.11 . In human, the Nek family is represented by 11 members that have been functionally associated to one of the three core functions established for this family in mammals: (1) centrioles/mitosis; (2) primary ciliary function/ciliop- athies; and (3) DNA damage response (DDR) (Meirelles et al., 2014). But new functions have been associated to this protein, as example, apoptotic functions (Hanchuk et al., 2015) and cell cycle (1 Mahjoub et al., 2005; Fletcher et al., 2005; Yin et al., 2003; Belham et al., 2003; Yissachar et al., 2006; Roig et al., 2002; Roig et al., 2005; Noguchi et al., 2004; O'Regan et al., 2009). No relation between Neks with polyglutamylation has been made until the present moment.

Here we describe the identification of Nek5 and TTLL4 protein partners by yeast two-hybrid screening that was confirmed by immunoprecipitation. Notably, stable cells expressing Nek5 regulates the activity of TTLL4 reducing the level of polyglutamylation while the knockdown or the kinase dead version increased the polyglutamylation profile after TTLL4 transfection. The *in vitro* polyglutamylation activity was also decreased in cells expression Nek5. The regulation of TTLL4 by Nek5 occurs in the C-termini region and the phosphorylation seems to be the mechanism of regulation. In a broader context, our analysis showed for the first time a mechanism for TTLL regulation.

MATERIALS AND METHODS

Stable cells, mutagenesis, and shRNA

The production of Hek293 cells stable expressing hNek5 and the silencing of Nek5 by shRNA (h) Lentiviral Particles (Santa Cruz Biotechnology, Inc) has been described previously (Hanchuk et al., 2015). Point mutations were introduced by QuikChange II Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA) to generate the Nek5-K33A, the inactive version of Nek5. Mutation was confirmed by DNA sequencing.

Yeast two-hybrid screening

The cDNA encoding the C-terminus of hNek5 (a.a. 260–708) was cloned in the pGBKT7. The yeast two-hybrid screen was performed following the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech Laboratories, Inc., Mountain View, CA) according to the manufacturer's instructions. Selective medium absent of tryptophan, leucin, adenin and histidin but containing aureobasidin antibiotic and X-α-Gal was used to screen interactors from human universal cDNA library. To identify the "preys" genes, the DNA was extracted and sequenced.

DNA Transfection

TTLL genes amplified from mouse brain or testis cDNA were previously cloned in vector containing a C-terminal EYFP tag (van Dijk et al., 2007). Expression plasmids were transfected using JetPEI (Polyplus transfection) or homemade PEI.

Immunoprecipitation

Cells were lysed in RIPA buffer with protease inhibitor cocktail (Roche Applied Science, Mannhein, Germany), subjected to freeze/thaw, submitted to three cycles of 5 minutes in an ultrasound bath (UltraSonic Clear 750, UNIQUE) for complete pellet resuspension and centrifuged at 20,000 x g for 30 min at 4°C. Protein concentration was determined by the method of Bradford (Bradford, 1976). The supernatants were used for immunoprecipitation as described by Chen, et al. (1989). Briefly, the supernatant was added to GFP-Trap® (ChromoTek GmbH, Germany) or anti-FLAG coupled to agarose beads and incubated overnight. Beads were collected, washed five times with wash buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA) and then elute with 2 x SDS-sample buffer (120 mM Tris/Cl pH 6.8; 20% glycerol; 4% SDS, 0.04% bromophenol blue; 10% β-mercaptoethanol). The proteins were immunoblotted to Immobilon-P membrane (Millipore Corp., Bedford, MA and probed with anti-Nek5 antibody. Blots were developed using an ECL chemiluminescence kit (Amersham Biosciences).

Immunoblotting analysis

50 μg of protein were separated by SDS-PAGE and blotted onto Immobilon-P membrane (Millipore Corp., Bedford, MA). In the case of mammalian tubulin was used special protocol, as in Edd. et al., 1987. The protein bands were probed by using the proper primary antibodies diluted in 3% bovine serum albumin blocking solution: mouse anti-GFP (1:1000, Sigma), mouse anti-Nek5 (1:500, Santa Cruz Biotechnology) and rabbit anti-TTLL4 (1:500, Novus Biologicals). The mouse GT335 (anti-polyglutamylation, 1:1000) and mouse 12G10 (anti-tubulin, 1:500) were produced by Janke's group (Wolff et al., 1992, van Dijk et al., 2007) and were used at 1:1000 dilutions. Detection of the bands were performed with HRP-labeled anti–rabbit or anti–mouse IgG (1:5,000; Sigma) followed by the chemiluminescent ECL Western Blotting System (ECL Western blot detection kit; GE Healthcare). Protein bands were quantified using the ImageJ software (National Institutes of Health, USA).

In Vitro Polyglutamylation Assay

Stable or silenced cell extracts were transfected with the active or an enzymatically inactive version of TTLL4 (E901G) and TTLL5. The cell extract was used for the determination of polyglutamylase activities as previously described (Janke et al., 2005; Regnard et al., 1998). Cells were resuspended in MEM buffer (50 mM Mes/NaOH, pH 6.8, 2 mM EGTA, 1 mM $MgCl_2$) buffer 0.2% NP-40 containing protease inhibitors). Reaction mixtures (50mM Tris-HCl [pH 9.0], 400 mM ATP, 2.4 mM MgCl2, 500 μM DTT, 4,6 μM taxol, 8 mML–[3H]-glutamate (45–55 Ci/mmol, GE Healthcare) and 0.2 mg/ml tubulin were incubated at 30°C for 2 hr. Tubulins were prepared from adult mouse brains as described (Regnard et al., 1999). The samples were separated in SDS-PAGE gels, blotted onto nitrocellulose membrane, and stained with Ponceau. Quantification of the radioactivity incorporated was performed by scintillation counting, as described before (Regnard et al., 1998).

Mass Spectrometric Analysis

Proteins content of the GFP affinity-purified fraction were separated by SDS-PAGE and Coomassie stained. Bands on expected size of TTLL4 and TTLL5 were excised from the gel and submitted to in-gel trypsin or chymotrypsin digestion. Peptides were concentrated and analyzed by MS/MS on a Q-Tof II mass spectrometer (Micromass Ltd.,

Manchester, UK). Data analysis was performed with Mascot (Matrix Science Ltd., London, UK) against the NCBI (The National Center for Biotechnology Information) Database.

Flow Cytometry and sorting

Transfected HEK293T, stable-Nek and shRNA-Nek5 cells were collected and disassociated using trypsin. Cells were resuspended in saline solution (PBS), passed through a 0,45uM and analyzed by flow cytometry. Gates were created to separate single cells using the FSC and SSC paramenters followed by a new gate for YFP positive cells. The sorting was performed by separation in positive and negative YFP cells on BD-FACS Aria flow cytometer using FACS Diva 6.0 software and data analyzed using FlowJo (Tree Star Inc., Ashland, OR, USA). The polyglutamylation analysis was done in BD-FACSCanto flow cytometer and the median of the Alexa-568-Mouse peak (polyglutamylation level) was generated solely with the positive YFP cells.

RESULTS

Yeast two-hybrid screening and immunoprecipitation identified TTLL4 as a new partner of Nek5.

The functions of Nek5 are little known. To the understanding of their interaction partners and their possible functions the yeast two-hybrid assay were performed with Matchmaker GAL4 system (Clontech Laboratories, Inc) using the C-terminal of Nek5 as bait fused to Gal4-DNA binding domain (DNA-BD). A cDNA human library were expressed fused to Gal4-activation domain (AD; Fields & Song, 1989; Chien *et al.*, 1991). After the protein-protein interaction, four independent transcription factors of reporter genes were activated (*AUR1-C*, *ADE2*, *HIS3*, e *MEL1*). The growth in selective medium allowed the identification of TTLL4 as Nek5 partner (fig. 1 a and b). By immunoprecipitation assay was possible to detect the co-immunoprecipitation of endogenous TTLL4 with Nek5 ful lenght fused to TTL4.

TTLLs are the effector proteins in polyglutamylation of tubulins. As Nek5 and TTL4 interact it was suggested a regulation of TTLL4 by Nek5. To confirm this, stable cells expressing Nek5 (Nek5) full length or the inactive version (kinase dead – Nek5-KD), as well as cells silenced for Nek5 (shRNA-Nek5) were transfected with TTLL4 and TTLL5 and the polyglutamylation profile was accessed by western blot. In the figure 1D is possible

to observe that in the presence of Nek5 the levels of polyglutamylation reduced drastically. The balance of alfa and beta tubulin also was altered by the presence of Nek5 and TTLL4 or TTLL5. shRNA-Nek5 and Nek5-KD cells transfected with TTLL4 showed exacerbated levels of polyglutamylation in β-tubulins when compared to Nek5 cells, but an increased on α-tubulin where also observed (Figure 1E). Although only Nek5 have been identified by yeast two-hybrid screening, the potentiating effects of Nek5 after transfection with TTLL4 were also observed with TTL5, suggesting that, Nek5 may play a role in other members of the TTLL's Family (Figure 1D and E). Beside this, polyglutamylation is not restricted to tubulin, it was shown to occur on nucleosome assembly proteins, NAP1 and NAP2 (Regnard et al., 2000), and probably on several yet unidentified proteins. In the figure 1, polyglutamylation of other proteins have been also altered in the absence of Nek5 and presence of TTLL4 or TTL5.

The presence of Nek5 reduce the levels of polyglutamylated proteins

The putative catalytic domain TTLL4, previously referred to as TTL domain, is subdivided two groups, one common to all TTLLs and TTL, now called ''core TTL domain,'' and the second conserved region upstream of it is now the ''extended TTL domain'' (Figure 2a) there is also necessary for TTLL4 activity the required region. Except for the mTTLL4 C606-1193 construction all the other truncations did not affect the activity of polyglutamylases (Van Dijk et al., 2007) but the presence of Nek5 was fundamental for this (Figure 2). To analyze the minimum sequence for Nek5 regulation truncated isoforms of TTLL4 were transfected in Nek5, Nek5-KD or shRNA-Nek5 cells (Fig. 2a-D). In the figure 2 it is possible to observe that Nek5 is capable to reduce the levels of polyglutamylation even in the smallest fragment suggesting that the regulation by Nek5 occur in the C-terminal region. The sequence interacting with Nek5 in the yeast two hybrid screening corresponds to the aa. 985 to 1189 in the C-terminal region. According to Uniprot TTLL4 is phosphorylated on S691 an S696 residues and using the Kinase-specific Phosphorylation Site Prediction GPS 2.1 several residues could be phosphorylated by Nek proteins by the aminoacids residues with the highest score are S960, S177, S946 and S912 (Supplementar data 1 –S1). By TTLL4 transfection is possible to observe several of non-tubulin proteins polyglutamylated in Nek5-KD and shRNA-Nek5 but for TTLL5 the differences are more pronounced in tubulins (Fig 2c and d). The transfection of TTLLs in

cells with active Nek5 did not alter the levels of polyglutamylation (Figure 2b).

In the presence of Nek5 TTL4 and TTLL5 are phosphorylated in several residues

The previous data suggests that Nek5 is capable to phosphorylate TTLL4 inhibiting its activity. To identify the phosphorylated residues in TTLL4 and TTLL5 stable cells expressing Nek5 and shRNA-Nek5 cells were transfected with EYFP-TTLL4 and EYFP-TTLL5. Nek5 and shRNA-Nek5 cells were transfected with TTLL4 and TTLL5. As the efficiency of the transfection is weak, transfected cells were submitted to sorting in flow cytometry to collect separately the cells expressing EYFP-TTLL4 or EYFP-TTLL5 and the negative cells. The positive and negative cells were lysed and the proteins separated by SDS-PAGE and analyzed by western blot. Cells expressing TTLL4 showed as expected, an increase in polyglutamylation profile in cells knockdown for Nek5, it is also possible to observe that negative cells did not show any polyglutamylation activity (Figure 3a). Using anti-GFP (TTLL4 staining), Stable cells expressing hNek5 and that were positive to EYPF-TTLL4 showed two closely located bands around the expected position, indicating phosphorylation (Fig. 3a). The presence of two nearby bands indicates phosphorylation or another kind of post-translational modification. As the second band is weak in the absence of Nek5, is suggested an inhibitory phosphorylation of TTLL4 by Nek5 (Fig. 3a). To analyze the residues phosphorylated in EYFP-TTLL4 and EYFP-TTLL5, the YFP positive cells sorted by flow cytometry were lysated and immunoprecipitated using anti-GFP beads and the elute fractions were separated by SDS-PAGE and he gel stained with coomassie blue (Fig. 3b). The bands in the height of TTLL4 or TTLL5 were cut, de-stained, submitted to trypsin or chymotrypsin digestion and analyzed by mass spectrometry. The phosphosites are represented in the figure 3C.

The activity of TTLL4 is highly regulated through phosphorylation

After the detection of phosphorylated sites it was generated phosphomimetic mutants to evaluate the capacity of phosphorylation to regulate the activity of TTLLs. The residues S912, T968, S1136 and S1139 of EYFP-TTLL4 were mutated to glutamic acid (E) and aspartic acid for S1139, mimicking the phosphorylation. The serine residues S1136 and S1139 were also mutated to alanine, blocking the phosphorylation. The phosphosites S281 and S1042 identified by mass spectrometry were also mutated to glutamic acid (S281E and S1042E). The mutants were transfected into Hek293T, Nek5-stable and shRNA-Nek5 cells.

The polyglutamylation level was analyzed by flow cytometry after staining of the cells with anti-GT335 and alexa-568. Only the positive cells for YFP were used in the identification of the level of polyglutamylation. As expected all the constructions transfected in shRNA-Nek5 cells showed pronounced levels of polyglutamylation when compared to stable-Nek5 and Hek293T cells (Fig 4A). The more pronounced effect observed was the decrease on TTLL4 activity when S1136 was mutated to alanine (S1136A) in Hek293T cells (Fig. 4B), indicating that TTLL4 may be regulated by phosphorylation. The shRNA-Nek5 cells showed reduced level of polyglutamylation when transfected with EYFP-TTLL4-S1136A when compared to the other construction, but no statistical differences were observed to the control group (EYFP-TTLL4). However, when transfected in stable-Nek5 cells all the constructs for TTLL4 presented similar results in the polyglutamylation level (Fig. 4D) and no effects were observed in S1136A. The phosphosites of EYFP-TTLL5 was also investigated. Serines 281 and 1042 were mutated to glutamic acid (S281E and S1042E) and transfected in the presence of Nek5. Besides the fact that shRNA-Nek5 cells showed higher levels of polyglutamylation in general, both S281E and S1042E showed more polyglutamylation in shRNA-Nek5 and stable-Nek5 cells (Fig. E-H). Together, these data may suggest that the regulation of TTLL4 by Nek5 occur through unknown residues or in complex with another protein. To answer these questions new experiments need to be done.

DISCUSSION

Tubulin polyglutamylation occurs at C-termini of both α and β tubulins by the addition of glutamate residues (van Dijk et al., 2007; Ikegami et al., 2006; Janke et al., 2005; Edde et al., 1990). Is suggested that polyglutamylation anchors MAPs and molecular motors (Janke & Bulinski, 2011) or can acts as a signal for a selective group of MAPs to associate with MTs for specific cellular functions clearly show selective association of the centriole and spindle-associated protein (CSAP) with polyglutamylated tubulin (Becker et al., 2012).

Enzymes that regulate the polyglutamylation have only been discovered recently and in total thirteen TTLL proteins and TTL were identified (van Dijk et al., 2007; Janke et al., 2005). TTLL4, 5, 6, 7, 11, and13 are involved on tubulin polyglutamylation activity when over-expressed in mammalian cells. TTLL5, 6, 11, and 13 prefer α –tubulin, while

TTLL4 and 7 prefer β -tubulin (Janke et al., 2005; Ikegami et al. 2006; van Dijk et al. 2007). TTLL12 has an increased expression in epithelial cells of prostate gland (Wasylyk et al., 2010), can delay cell cycle progression from G2/M phase to G1 (Brants et al., 2012) and is considered an antigen in cancer patients (Massoner et al., 2012). TTLL4 is a know β-tubulin modifier (van Dijk, 2007) which its increased activation causes polyglutamylation of non-tubulin substrates and remodels chromatin (van dijk et al., 2007; Kashiwaya et al., 2010). TTLL4 showed increased levels of expression in pancreatic cancer cells and its knockdown reduced their growth (Kashiwaya et al., 2010).

Mutations of Nek family members have been associated to development of ciliopathies and cancer (Tsunoda et al., 2009; Marina e Saavedra, 2014; Liu et al., 2014; McHale et al., 2008; Takeno et al., 2008; Capra et al., 2006; Bowers et al., 2004; Cao et al., 2012. In this study we showed that Nek5 interacts with TTLL4 by Y2H screening and immunoprecipitation assay.

Also we show that in the presence of Nek5, the transfection of TTLL4 do not increase the levels of polyglutamylation, suggesting an inhibition of activity. The fine regulation of polyglutamylation profile contributes to defects on ciliary beating (Million et al., 1999) and decrease on polyglutamylation- conjugating enzymes reduces polyglutamylation on tubulins and the cilia beating in *Chlamydomonas reinhardtii* (Kubo et al., 2010), *Tetrahymena* (Suryavanshi et al., 2010), and zebrafish (Pathak et al., 2007). On the other hand overexpression of TTLLs involved on β-tubulin polyglutamylation results in inhibition of ciliary beating (Janke et al., 2005). Kinase dead and shRNA-Nek5 cells showed increased levels of polyglutamylation when transfected with TTLL4. But not only tubulins presented increased levels of polyglutamylation, TTLL4 is a well know TTLL member involved on polyglutamylation of different substrates (van Dijk et al., 2008). Nucleosome assembly proteins (NAPs) and end binding protein 1 (EB1) also are modified by polyglutamylation (Regnard et al., 2000; van Dijk, 2008).

The relation between Neks and TTLLs is favored by localization. Both are located and/or have functions related to cilium and centrosomes. Neks are extensively related to centrosomes and primary cilium (Patil etal., 2013; Hilton etal., 2009; Mahjoub et al., 2005; Lee et al., 2014; Sdelci et al., 2012; Kim et al., 2011; Sdelci et al., 2011; Habbig et al., 2012; Zalli et al., 2012). Nek1 and Nek8 have a ciliary function and mutations in its genes

lead to polycystic kidney disease (Guay-Woodford et al., 2003; Upadhya et al., 2000). NEK1, has also previously been shown to localize near centrosomes and to play a role in centrosomal stability and ciliogenesis (Mahjoub et al., 2005; White et al., 2008). Indeed, centrosomes and basal bodies are highly polyglutamylated with long Glu side chainsand this modifications are related to function in generating or maintaining the stability of the organelle (Bobinnec et al., 1998; Geimer et al., 1997).

Aberrant glutamylation are related to different diseases. Disturbed polyglutamylation and tyrosination/detyrosination are mediators of cancer progression (Soucek et al., 2006; Kato et al., 2004) and hiperglutamylation has been associated to neurodegeneration in mouse models and can be reversed by TTLL1 inhibition (Rogowski et al., 2010). In this work we showed that the serine 1136 of TTLL4 is important for its activity. Inhibitors of tubulin tyrosination and deacetylation are not only promising therapeutic candidates for cancer treatment (Dal Piaz et al., 2009; Lane et al., 2009) but also have contributed to our knowledge about the biological roles of these enzymes. So, polyglutamylase inhibitors/ regulators could also work similarly.

Westermann and colleagues (2002) reported for the first time an CfNek present in a highly purified preparation of tubulin polyglutamylase. Isolated cytoskeletons from *Crithidia fasciculate* was used to purify glutamylation activity by ATP-affinity chromatography and the CfNek was copurified. However, the expression of CfNek in *E. coli* or insect cells did not show phosphorylation or glutamylation activity that could be due to wrong folding, the lack of some activation step or the necessity to work in a complex (Westermann et al., 2002). Despite to indicate the role of CfNek in polyglutamylation, it was not showed any directly catalyse of glutamylation suggesting that the role of CfNek is similar to Nek5 that alters the level of polyglytamylation through phosphorylation of effector enzymes (TTLL4 for example).

The relation between Neks and members of TTLL family needs to be more explored. The regulation of TTLL4 by Nek5 and the fact that centrosome stability depends upon tubulin polyglutamylation (Bobinnec et al., 1998b) and Nek2 kinases from different organisms are involved in centrosome maturation and integrity (Fry et al., 1998; Uto and Sagata, 2000) indicates that this two family of proteins are more related to the hypothesized until the moment.

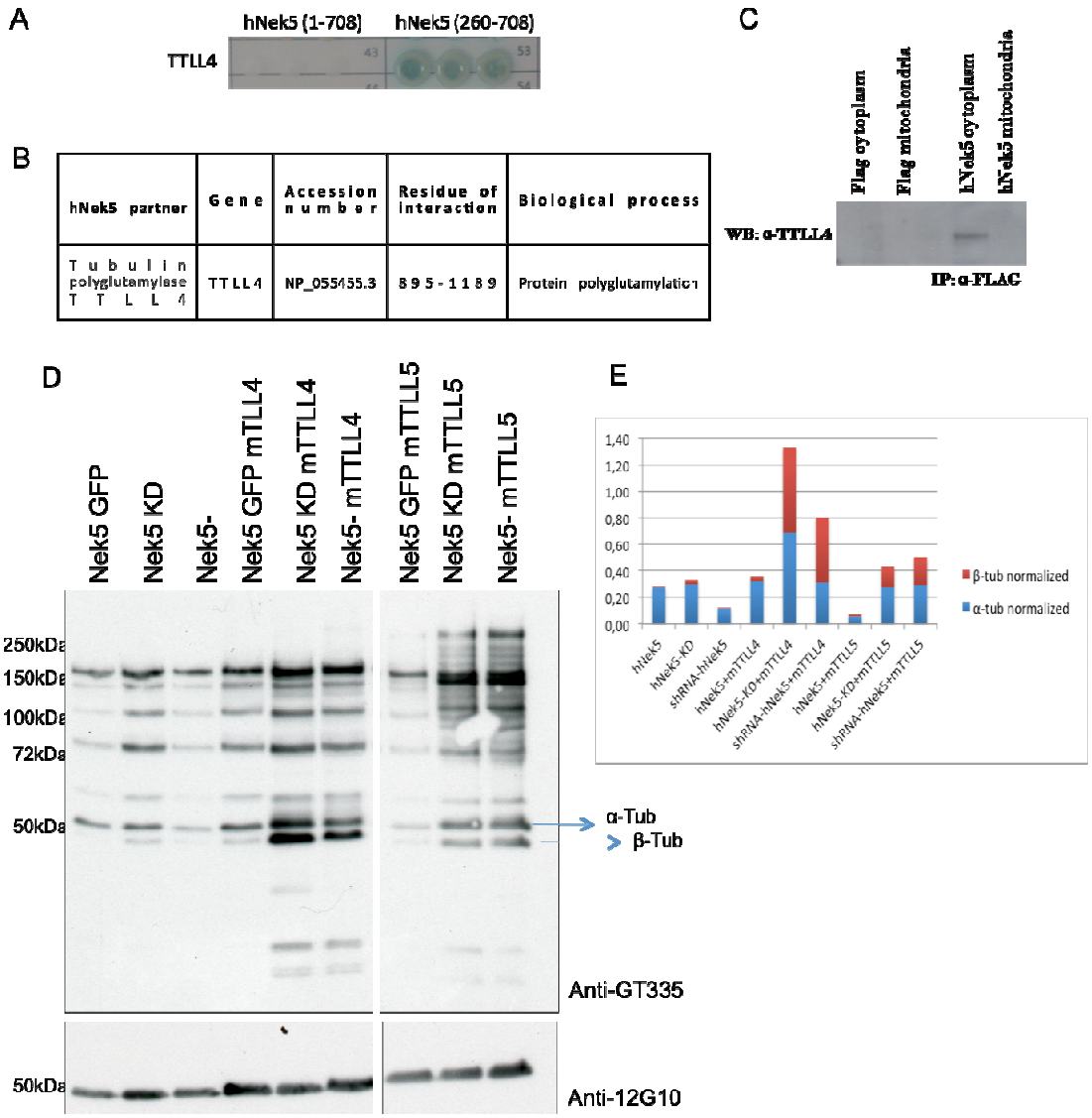


Figure 1: Nek5 interacts with TTLL4. a) The colonies grew in selective medium SD-WLAH with aureobasidin and X-α-Gal. In addition to the reporter gene Auri-C were also activated the genes MEL1, ADE2 His3 as indicators of interaction between the proteins. b) The table indicates the identified region of the TTLL4 by yeast two-hybrid screening. The C-terminal region of hNek5 (260-708) was positive to interact with the region of TTLL4 (895-1189aa). c) Stable cells expressing Nek5-FLAG were induced with 2ug/mL of tetracycline and fractionated in cytoplasm and mitochondria. 1ug of proteins were used to immunoprecipitate Nek5 using an anti-FLAG resin, after elution the proteins were separated by SDS-PAGE and blotted to nitrocellulose membrane. TTLL4 co-immunoprecipitated with Nek5 as accessed with anti-TTLL4 antibody.

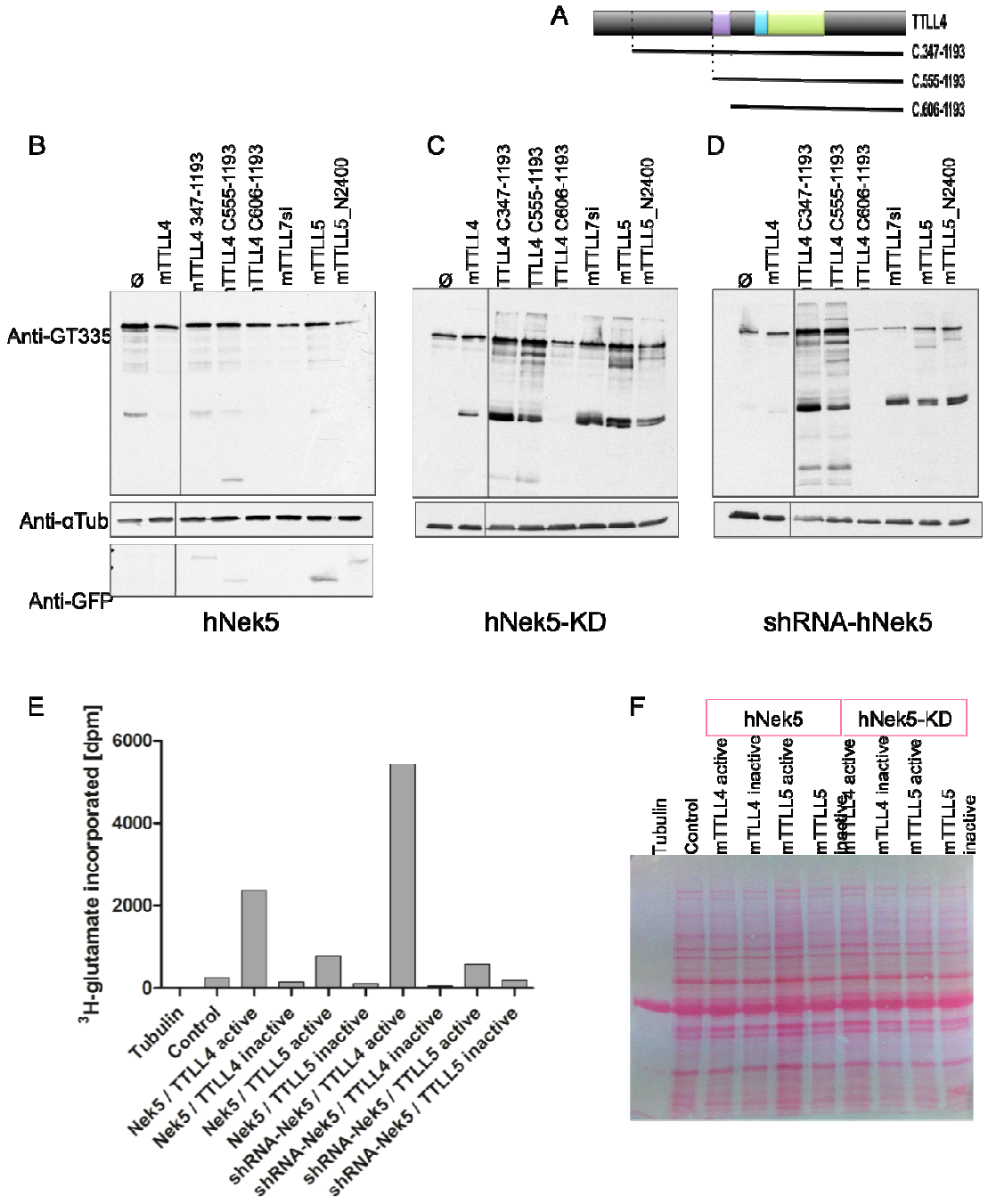
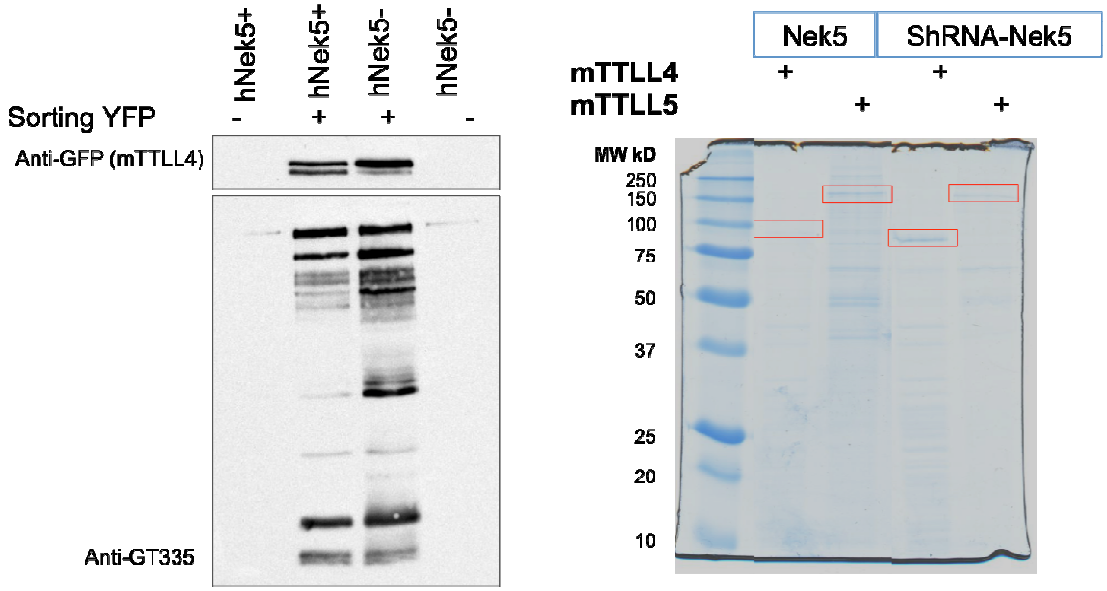


Figure 2: The effect of Nek5 in different truncations TTLL4 and its corresponding enzymatic activity was determined. a) Schematic representation of the TTLL constructs used. The sequences expressed for each truncated version are depicted as black bars underneath the pictograms of the full-length TTLL proteins. Domains dispensable or required for autonomous activities are shown. B, c and d) Analysis by western blot of truncations TTLL4 and 5 in polyglutamylation in the presence of stable cells for Nek5 and silenced for this protein. E) In vitro experiment for polyglutamylase activity: Tubulins from brain were subjected to in vitro polyglutamylation assays with extracts from Nek5 and shRNA-Nek5 cells expressing the respective TTLL-

EYFP proteins. F) Membrane used on in vitro polyglutamylase activiy stained with Ponceau showing the loading of proteins.



Protein	Phosphorylated Residue in hNek5	Phosphorylated Residues in shRNA-hNek5
mTTLL4	S912, S960, T968, S1136, S1139	T813, Y815, S912, T968, S1136, S1139
mTTLL5	S281, S1042	S1123

Figure 3: Nek5 decreased TTLL4 activity. shRNA-Nek5 and stable-Nek5 cells transfected with EYFP-TTLL4 or EYFP-TTLL5 were sorted by flow cytometry. A) cells transfected with EYFP-TTLL4 were lysed and analyzed by western-blot. B) The protein extract from EYFP positive cells were immunoprecipitated using GFP-beads for TTLL5 purification. C) Phosphorylated residues detected in TTLL4 and TTLL5.

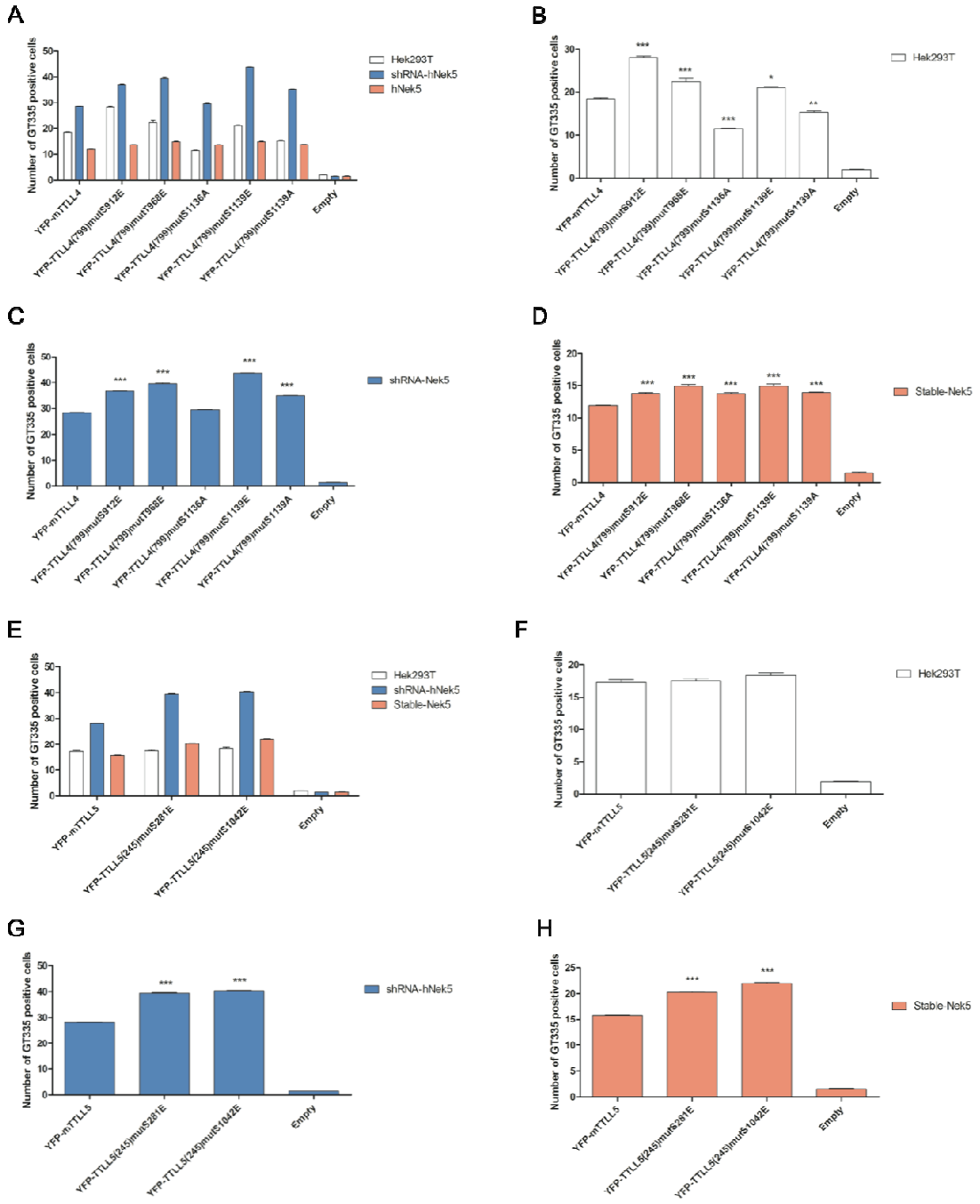


Figure 4: The serine 1136 is important for TTLL4 activity. Based on the residues identified by mass spectrometer, phosphomimetic mutants were generated. Hek293T, shRNA-Nek5 and stable-Nek5 cells transfected with EYFP-TTLL4 and EYFP-TTLL5 were stained with anti-GT335 and anti-mouse Alexa®568 and analyzed by flow cytometer. The EYFP-TTLL4 mutants were transfected in Hek293T (B), shRNA-Nek5 (C) and stable-Nek5 cells (D). EYFP-TTLL5 mutants were transfected in Hek293T (F), shRNA-Nek5 (G) and stable-Nek5 cells (H).

REFERENCES

Belham, C.; Roig, J.; Caldwell, J. A.; Aoyama, Y.; Kemp, B. E.; Comb, M.; Avruch, J. A. (2003). Mitotic cascade of NIMA family kinases. Nercc1/Nek9 activates the Nek6 and Nek7 kinases. J. Biol. Chem. 278(37): 34897–34909.

Blum ES, Schwendeman AR, Shaham S. (2013). PolyQ disease: misfiring of a developmental cell death program?. Trends Cell Biol. 23(4):168-74

Bobinnec, Y.; Khodjakov, A.; Mir, L. M.; Rieder, C. L.; Eddé, B. and Bornens, M. (1998). Centriole Dissassembly in vivo and its effect on centrosome structure and function in vertebrate cells. J. Cell Biol. 143: 1575-1589.

Bonnet, C., Boucher, D., Lazereg, S., Pedrotti, B., Islam, K., Denoulet, P. and Larcher, J.C. (2001) Differential Binding Regulation of Microtubule-associated Proteins MAP1A, MAP1B, and MAP2 by Tubulin Polyglutamylation. J Biol Chem, 276, 12839-12848.

Boucher, D., Larcher, J.C., Gros, F. and Denoulet, P. (1994) Polyglutamylation of tubulin as a progressive regulator of in vitro interactions between the microtubule-associated protein Tau and tubulin. Biochemistry, 33, 12471-12477.

Bowers, A.J. and Boylan, J.F. (2004). Nek8, a NIMA family kinase member, is overexpressed in primary human breast tumors. Gene. 328:135-42.

Brants, J.; Semenchenko, K.; Wasylyk, C.; Robert, A.; Carles, A.; Zambrano, A.; Pradeau-Aubreton, K.; Birck, C.; Schalken, J.A.; Poch, O.; Mey, J.; Wasylyk, B. (2012). Tubulin tyrosine ligase like 12, a TTLL family member with SET- and TTL-like domains and roles in histone and tubulin modifications and mitosis. PLoS ONE. 7: e51258.

Cao, X.; Xia, Y.; Yang, J.; Jiang, J.; Chen, L.; Ni, R.; Li, L.; Gu, Z. Clinical and biological significance of never in mitosis gene A-related kinase 6 (NEK6) expression in hepatic cell cancer. Pathol Oncol Res. 18(2): 201-7.

Capra, M.; Nuciforo, P. G.; Confalonieri, S.; Quarto, M.; Bianchi, M.; Nebuloni, M.; Boldorini, R.; Pallotti, F.; Viale, G.; Gishizky, M. L.; Draetta, G. F.; Di Fiore, P. P. Frequent alterations in the expression of serine/threonine kinases in human cancers. Cancer Res. 66(16): 8147-54.

Dal Piaz, F.; Vassallo, A.; Lepore, L.; Tosco, A.; Bader, A.; De Tommasi, N. (2009). Sesterterpenes as tubulin tyrosine ligase inhibitors. First insight of structureactivity relationships and discovery of new lead, J. Med. Chem. 52: 3814–3828.

Eddé, B.; Rossier, J.; Le Caer, J.P.; Desbruyères, E.; Gros, F.; Denoulet, P. (1990). Posttranslational glutamylation of alpha-tubulin. Science. 247(4938): 83-5.

Ersfeld, K., Wehland, J., Plessmann, U., Dodemont, H., Gerke, V., and Weber, K. (1993). Characterization of the tubulin-tyrosine ligase. J Cell Biol 120, 725-732.

Fletcher, L.; Cerniglia, G. J.; Yen, T. J.; Muschel, R. J. (2005). Live cell imaging reveals distinct roles in cell cycle regulation for Nek2A and Nek2B. Biochim. Biophys. Acta.1744(2): 89–92.

Fry, A.M.; Mayor, T.; Meraldi, P.; Stierhof, Y.D.; Tanaka, K.; Nigg, E.A. C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle-regulated protein kinase Nek2. J Cell Biol. 141: 1563-1574

Geimer, S.; Teltenkötter, A.; Plessmann, U.; Weber, K.; Lechtreck, K.F. (1997). Purification and characterization of basal apparatuses from a flagellate green alga. Cell Motil Cytoskeleton. 37(1): 72-85.

Guay-Woodford, L.M. (2003) Murine models of polycystic kidney disease: molecular and therapeutic insights. Am. J. Physiol. Renal Physiol. 285: 1034–1049.

Habbig, S.; Bartram, M.P.; Sägmüller, J.G.; Griessmann, A.; Franke, M.; Müller, R.U.; Schwarz, R.; Hoehne, M.; Bergmann, C.; Tessmer, C.; Reinhardt, H.C.; Burst, V.; Benzing, T.; Schermer, B. (2012). The ciliopathy disease protein NPHP9 promotes nuclear delivery and activation of the oncogenic transcriptional regulator TAZ. Hum Mol Genet. 21(26): 5528-38.

Hilton, L.K.; White, M.C.; Quarmby, L.M. (2009). The NIMA-related kinase NEK1 cycles through the nucleus. Biochem Biophys Res Commun. 389(1): 52-6.

Ikegami, K.; Mukai, M.; Tsuchida, J.I.; Heier, R.L.; Macgregor, G.R. and Setou, M. (2006) TTLL7 is a mammalian beta-tubulin polyglutamylase required for growth of MAP2-positive neurites. J Biol Chem. 281: 30707-30716.

J. van Dijk, K. Rogowski, J. Miro, B. Lacroix, B. Eddé, C. Janke. (2007). A targeted multienzyme mechanism for selective microtubule polyglutamylation, Mol. Cell. 26: 437–448.

Janke, C; Bulinski, JC. (2011) Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions, Nat. Rev. Mol. Cell Biol. 12: 773–786.

Janke, C.; Rogowski, K.; Wloga, D.; Regnard, C.; Kajava, A.V.; Strub, J.M.; Temurak, N.; van Dijk, J.; Boucher, D.; van Dorsselaer, A.; Suryavanshi, S.; Gaertig, J.; Eddé, B. (2005). Tubulin polyglutamylase enzymes are members of the TTL domain protein family. Science. 308: 1758–1762.

Kashiwaya, K.; Nakagawa, H.; Hosokawa, M.; Mochizuki, Y.; Ueda, K.; Piao, L.; Chung, S.; Hamamoto, R.; Eguchi, H.; Ohigashi, H.; Ishikawa, O.; Janke, C.; Shinomura, Y.; Nakamura, Y. (2010). Involvement of the tubulin tyrosine ligase-like family member 4 polyglutamylase in PELP1 polyglutamylation and chromatin remodeling in pancreatic cancer cells, Cancer Res. 70: 4024–4033.

Kato, C.; Miyazaki, K.; Nakagawa, A.; Ohira, M.; Nakamura, Y.; Ozaki, T.; Imai, T.; Nakagawara, A. (2004). Low expression of human tubulin tyrosine ligase and suppressed tubulin tyrosination/detyrosination cycle are associated with impaired neuronal differentiation in neuroblastomas with poor prognosis, Int. J. Cancer 112: 365–375.

Kim, S.; Kim, S.; Rhee, K. (2011). NEK7 is essential for centriole duplication and centrosomal accumulation of pericentriolar material proteins in interphase cells. J Cell Sci. 124(Pt 22):3760-70.

Kubo, T.; Yanagisawa, H.; Yagi, T.; Hirono, M.; Kamiya, R. (2010). Tubulin polyglutamylation regulates axonemal motility by modulating activities of inner-arm dyneins, Curr. Biol. 20: 441–445.

Lane, A.A.; Chabner, B.A. Histone deacetylase inhibitors in cancer therapy, J. Clin. Oncol. 27: 5459–5468.

Larcher, J.C., Boucher, D., Lazereg, S., Gros, F. and Denoulet, P. (1996) Interaction of kinesin motor domains with alpha- and beta-tubulin subunits at a tau-independent binding site. Regulation by polyglutamylation. J Biol Chem, 271, 22117-22124.

Lechtreck, K.F., and Geimer, S. (2000). Distribution of polyglutamylated tubulin in the flagellar apparatus of green flagellates. Cell Motil Cytoskeleton, 47, 219-235.

Lee, M.Y.; Moreno, C.S.; Saavedra, H.I. (2014). The E2F activators signal and maintain centrosome amplification in breast cancer cells.

Liu, X; Gao, Y; Lu, Y; Zhang, J; Li, L and Yin, F. (2014). Upregulation of NEK2 is associated with drug resistance in ovarian cancer. Oncol Rep. 31(2):745-54.

Mahjoub, M.R.; Trapp, M.L.; Quarmby, L.M. (2005). NIMA-related kinases defective in murine models of polycystic kidney diseases localize to primary cilia and centrosomes. J Am Soc Nephrol. 16(12):3485-9.

Marina, M.; Saavedra, H. I. Nek2 and Plk4: prognostic markers, drivers of breast tumorigenesis and drug resistance. Front Biosci (Landmark Ed). 19: 352-65.

Massoner, P.; Lueking, A.; Goehler, H.; Höpfner, A.; Kowald, A.; Kugler, K.G.; Amersdorfer, P.; Horninger, W.; Bartsch, G.; Schulz-Knappe, P.; Klocker, H. (2012). Serumautoantibodies for discovery of prostate cancer specific biomarkers. Prostate. 72: 427–436.

Mchale, K.; Tomaszewski, J. E.; Puthiyaveettil, R.; Livolsi, V. A.; Clevenger, C. V. Altered expression of prolactin receptor-associated signaling proteins in human breast carcinoma. Mod Pathol. 21(5): 565- 71.

Million, K.; Larcher, J.; Laoukili, J.; Bourguignon, D.; Marano, F.; Tournier, F. (1999). Polyglutamylation and polyglycylation of alpha- and beta-tubulins during in vitro ciliated cell differentiation of human respiratory epithelial cells. J Cell Sci. 112 (Pt 23):4357-66.

Noguchi, K.; Fukazawa, H.; Murakami, Y.; Uehara, Y. (2004). Nucleolar Nek11 is a novel target of Nek2A in G1/S-arrested cells. J. Biol. Chem. 279(31): 32716–32727.

O'Connell MJ, Krien MJ, Hunter T. Never say never. The NIMA-related protein kinases in mitotic control. Trends Cell Biol 2003; 13: 221-228 [PMID: 12742165 DOI: 10.1016/

O'regan L, Blot J, Fry AM. Mitotic regulation by NIMA- related kinases. Cell Div 2007; 2: 25 [PMID: 17727698 DOI:10.1186/1747-1028-2-25] S0962-8924(03)00056-4]

Pathak, N.; Obara, T.; Mangos, S.; Liu, Y.; Drummond, I.A. (2007). The zebrafish fleer gene encodes an essential regulator of cilia tubulin polyglutamylation, Mol. Biol. Cell 18: 4353–4364.

Patil, M.; Pabla, N.; Ding, H.F.; Dong, Z. (2013). Nek1 interacts with Ku80 to assist chromatin loading of replication factors and S-phase progression.

Peter Bieling, Stefanie Kandels-Lewis, Ivo A. Telley, Juliette van Dijk, Carsten Janke, and Thomas Surrey. (2008). CLIP-170 tracks growing microtubule ends by dynamically recognizing composite EB1/tubulin binding sites. JCB. 183(7): 1223-1233

Redeker V, Levilliers N, Schmitter JM, Le Caer JP, Rossier J, Adoutte A, Bre MH (1994) Polyglycylation of tubulin: a posttranslational modification in axonemal microtubules. Science 266: 1688-1691.

Regnard, C., Desbruyeres, E., Denoulet, P. and Eddé, B. (1999) Tubulin polyglutamylase: isozymic variants and regulation during the cell cycle in HeLa cells. J Cell Sci, 112, 4281-4289.

Regnard, C.; Desbruyères, E.; Huet, J.C.; Beauvallet, C.; Pernollet, J.C.; Eddé, B. (2000). Polyglutamylation of nucleosome assembly proteins, J. Biol. Chem. 275: 15969–15976.

Rogowski, K.; van Dijk, J.; Magiera, M.M.; Bosc, C.; Deloulme, J.C.; Bosson, A.; Peris, L.; Gold, N.D.; Lacroix, B.; Grau, M.B.; Bec, N.; Larroque, C.; Desagher, S.; Holzer, M.; Andrieux, A.; Moutin, M.J. and Janke C. Cell. 143:564–578.

Roig, J.; Mikhailov, A.; Belham, C.; Avruch, J. (2002). Nercc1, a mammalian NIMA-family kinase, binds the Ran GTPase and regulates mitotic progression. Genes Dev. 16(13): 1640–1658.

Roig, J.; Groen, A.; Caldwell, J.; Avruch, J. Active Nercc1 protein kinase concentrates at centrosomes early in mitosis and is necessary for proper spindle assembly. Mol. Biol. Cell 2005, 16 (10), 4827–4840.

Sdelci, S.; Bertran, M.T.; Roig, J. (2011). Nek9, Nek6, Nek7 and the separation of centrosomes. Cell Cycle. 10(22): 3816-7.

Sdelci, S.; Schütz, M.; Pinyol, R. Bertran, M.T.; Regué, L.; Caelles, C.; Vernos, I.; Roig, J. (2012). Nek9 phosphorylation of NEDD1/GCP-WD contributes to Plk1 control of γ-tubulin recruitment to the mitotic centrosome. Curr Biol. 22(16): 1516-23.

Soucek, K; Kamaid, A; Phung, A.D.; Kubala, L.; Bulinski, J.C.; Harper, R.W.; Eiserich, J.P. (2006). Normal and prostate cancer cells display distinct molecular profiles of a-tubulin posttranslational modifications. Prostate. 66: 954–965.

Suryavanshi, S.; Eddé, B.; Fox, L.A.; Guerrero, S.; Hard, R.; Hennessey, T.; Kabi, A.; Malison, D.; Pennock, D.; Sale, W.S.; Wloga, D.; Gaertig, J. (2010). Tubulin

glutamylation regulates ciliary motility by altering inner dynein arm activity, Curr. Biol. 20: 435–440.

Takeno, A.; Takemasa, I.; Doki, Y.; Yamasaki, M.; Miyata, H.; Takiguchi, S.; Fujiwara, Y.; Matsubara, K.; Monden, M. Integrative approach for differentially overexpressed genes in gastric cancer by combining large scale gene expression profiling and network analysis. Br J Cancer. 99(8): 1307-15.

Tsunoda, N.; Kokuryo, T.; Oda, K.; Senga, T.; Yokoyama, Y.; Nagino, M.; Nimura, Y.; Hamaguchi, M. Nek2 as a novel molecular target for the treatment of breast carcinoma. Cancer Sci. 100(1): 111-6.

Upadhya, P., Birkenmeier, E.H., Birkenmeier, C.S. and Barker, J.E. (2000). Mutations in a NIMA-related kinase gene, Nek1, cause pleiotropic effects including a progressive polycystic kidney disease in mice. Proc. Natl Acad. Sci. USA. 97: 217–221.

Uto, K.; Nakajo, N. and Sagata, N. (1999) Two structural variants of Nek2 kinase, termed Nek2A and Nek2B, are differentially expressed in Xenopus tissues and development. Dev Biol. 208(2):456-64.

van Dijk, J.; Miro, J.; Strub, J.M.; Lacroix, B.; van Dorsselaer, A.; Edde, B.; Janke, C. (2008). Polyglutamylation is a post-translational modification with a broad range of substrates, J. Biol. Chem. 283: 3915–3922.

Verhey, K.J., and Gaertig, J. (2007). The Tubulin Code. Cell Cycle, 6, 2152-2160.

Yin, M. J.; Shao, L.; Voehringer, D.; Smeal, T.; Jallal, B. (2003). The serine/ threonine kinase Nek6 is required for cell cycle progression through mitosis. J. Biol. Chem. 278 (52): 52454–52460.

Yissachar, N.; Salem, H.; Tennenbaum, T.; Motro, B. (2006). Nek7 kinase is enriched at the centrosome, and is required for proper spindle assembly and mitotic progression. FEBS Lett. 580(27): 6489–6495.

Wasylyk, C.; Zambrano, A.; Zhao, C.; Brants, J.; Abecassis, J.; Schalken, J.A.; Rogatsch, H.; Schaefer, G.; Pycha, A.; Klocker, H.; Wasylyk, B. (2010). Tubulin tyrosine ligase like 12 links to prostate cancer through tubulin posttranslational modification and chromosome ploidy, Int. J. Cancer. 127: 2542–2553.

Westermann, S. and Weber, K. (2002). Identification of CfNek, a novel member of the NIMA family of cell cycle regulators, as a polypeptide copurifying with tubulin polyglutamylation activity in Crithidia. Journal of Cell Science 115(24): 5003-5012.

White, M.C.; Quarmby, L.M. (2008). The NIMA-family kinase, Nek1 affects the stability of centrosomes and ciliogenesis. BMC Cell Biol. 9:29.

Zalli, D.; Bayliss, R.; Fry, A.M. (2012). The Nek8 protein kinase, mutated in the human cystic kidney disease nephronophthisis, is both activated and degraded during ciliogenesis. Hum Mol Genet. 21(5):1155-71.

Discussão

As Neks representam a terceira maior família de cinases envolvidas na regulação do ciclo celular. Embora os membros desta família compartilhem alta similaridade em seu domínio catalítico, o domínio regulatório possui clássicas regiões de interação proteína-proteína, como a região *coiled–coil*. Dessa forma, a diversidade de substrato, bem como os diferentes processos biológicos aos quais estas proteínas estão relacionadas, se devem a diversidade do domínio regulatório (Artigo I). A identificação de parceiros de interação com cinases possui informação relativamente restrita na literatura devido a fraca e transiente interação entre as proteínas.

Neste trabalho é possível observar no artigo I uma revisão bibliográfica da família das Nek cinases e os principais avanços sobre cada um dos 11 membros da família das Neks. Em linhas gerais postulamos que cada membro da família deve estar envolvido em pelos menos um dos processos biológicos: função centriolar/mitose, cílio primário/ciliopatias e na resposta a dano de DNA. Além destas funções, a rede de interação entre as Neks e outras proteínas permitiram observar que as elas podem regular outros processos biológicos, tais como a morte celular. A Nek6 por exemplo interage com proteínas associadas ao processo de apoptose, como a proteína cinase serina/treonina PAK6 (PAK6), proteína cinase serina/threonina Sgk1 (SGK1) e o complexo DBIRD (Meirelles et al., 2010). A Nek1 também atua no processo apoptótico através da fosforilação do canal aniônico voltagem dependente (VDAC). VDAC é um complex poro que funciona como canal de voltage dependente de anion e como poro de permeabilidade que regula a liberação de citocromo c para o citoplasma, necessário para iniciar os eventos apoptótico (Chen et al., 2009).

Como a região regulatória é a principal região de interação das Nek cinases e seus substrates, foi utilizada a região regulatória de Nek5 (260-708 aa.) para o screening de parceiros por duplo híbrido. Foram obtidos 10 parceiros de interação com Nek5: MTX2, SNX6, c6orf225, DCAF10, CAP2, BCLAF1, SETDB1, TTLL4, TOPO2B e COX11. Experimentos de imunoprecipitação de Nek5 seguido por espectrometria de massas também revelaram diversos pontenciais alvos para Nek5. Dentre as proteína identificadas por Y2H, três apresentaram localização mitochondrial (COX11 e MX2) ou com função relacionada a mitochondria (BCLAF1). A tabela dos parceiros identificados encontra-se no anexo deste trabalho. No artigo II exploramos a interação entre Nek5 e estas proteínas

mitochondrias, bem como o papel de Nek5 no processo de morte cellular. Além da localização nuclear e citoplasmática, novas localizações estão sendo recentemente atribuídas as Neks. Nek1, já foi identificada como uma proteína mitocondrial e mostramos neste trabalho que Nek5 também está localizada nesta organela (Chen et al., 2009). Células expressando estavelmente Nek5 ou células silenciadas para a expressão desta proteína foram tratadas com a tapsigargina. Células silenciadas para Nek5 apresentaram uma morte acentuada após o tratamento com a tapsigardina e de taxas elevadas de formação de espécies reativas de oxigênio, sugerindo que Nek5 possa atuar como uma proteína anti-apoptótica, assim como o papel desenvolvido por Nek1. O papel de Nek5 na morte cellular pode ocorrer via interação entre Nek5 e suas presas, uma vez que MTX2, BCLAF1 e COX11 possuem papel relacionado a morte celular. MTX1 e MTX2 estão envolvidas na morte celular induzida por TNFα (Wang et al., 2001; Mazumder et al., 2007) e a depleção de MTX2 provoca uma deficiência na importação de VDAC e Tom40 (Abdul et al., 2000). BCLAF1 pode regular negativamente o processo de reparo mediado pelo complexo Ku70/p18-ciclinaE promovendo a apoptose (Zeviani et al., 1998). E em células silenciadas ou mutadas para Cox11 apresentaram hipersensibilidade a tratamentos com peróxido de hidrogênio (Pungartnik et al., 1999). Em linhas gerais, apresentamos no artigo II as primeiras evidências sobre o papel de Nek5 no processo de morte celular, uma vez que o único artigo existente na literatura sobre a Nek5 associa esta proteína com a diferenciação muscular após clivagem por Caspase-3 (Shimizu et al., 2013).

O interação entre Nek5 e TTLL4 também foi explorada em parceria com o Dr. Carsten Janke do Institut Curie. O artigo II apresenta os principais resultados obtidos desta caracterização de interação. A TTLL4 (tubulina poliglutamilase 4) está envolvida na iniciação da poliglutamilação. A poliglumatamilação é uma modificação pós traducional no qual cadeias laterais de glutamato são adicionadas à região C-terminal de proteínas (Eddé et al., 1990; Redeker et al., 1994). O papel da poliglutamilação no contexto celular ainda não está bem esclarecido, porém, acredita-se que esta modificação regule a interação entre proteínas. As tubulinas são os principais substratos para poliglutamilação principalmente em neurônios, células em divisão, midbodies, centrossomos e cílios e flagelos (xxx) e podem modular a associação dos microtúbulos com proteínas motoras (Bonnet *et al.*, 2001; Boucher *et al.*, 1994; Larcher *et al.*, 1996). Além das tubulinas, as proteínas TTLL4 e

TTLL5 são capazes de glutamilar diversas proteínas como PELP1 cuja glutamilação esta envolvida com o processo de remodelamento da cromatina em células de câncer pancreático (Kashiwaya et al., 2010). Após transfecção de TTLL4 em células expressando estavelmente Nek5 os níveis de proteínas poliglutamiladas reduziram drasticamente, ao passo que células silenciadas ou expressando estavelmente a versão *kinase dead* apresentaram níveis elevados de poliglutamilação. Células com Nek5 ou silenciadas foram transfectadas com TTLL4 e as células TTLL-positivas foram separadas por citometria de fluxo e analisadas por western blot. Na presença de Nek5 duas bandas foram observadas por western blot, sugerindo fosforilação. As bandas relativas a TTLL4 foram analisadas por espectrometria de massas e diversos resíduos foram identificados como fosforilados. Em linhas gerais, demonstramos neste trabalho que a fosforilação de TTLL4 por Nek5 pode inativar esta proteína e o processo de poliglutamilação de tubulinas e outros substratos. A poliglutamilação está envolvida com a estabilidade de centrossomos (Bobinnec et al., 1998b) e a Nek2 envolvida na sua maturação e integridade (Fry et al., 1998; Uto and Sagata, 2000) sugerindo que estas famílias de proteínas estão mais relacionadas do que hipotetizado até o momento. A relação entre os membros das famílias Nek e TTLL precisam ser melhor explorados porém com este trabalho o primeiro passo já foi dado no sentindo de estabelecer uma nova função para a família das Neks.

Este projeto direcionou-se portanto, no sentido de desvendar funcionalmente a proteína humana hNek5. Em linhas gerais, este projeto identificou potenciais vias de sinalização da Nek5 humana, com ênfase na morte celular e no processo de poliglutamilação. As informações obtidas por este trabalho são as primeiras sobre a função de Nek5 no contexto celular e contribuem, não somente para o campo da biologia molecular, como para futuros estudos visando o maior conhecimento sobre o membros da família das Neks.

Conclusões

Muitos avanços tem sido realizados no intuito de expandir o conhecimento sobre os membros da família das Neks, entretanto, alguns membros desta família, principalmente a Nek5, continuam sendo negligenciados quanto às suas funções. No presente trabalho foi realizada a caracterização funcional de Nek5. Foram realizados ensaios funcionais, moleculares e bioquímicos para caracterizar os parceiros de interação da Nek5 e as funções atribuídas a esta proteína.

Em linhas gerais este trabalho conclui que:

- No artigo de revisão foi realizada uma análise detalhada atual sobre cada uma das 11 Neks e seu envolvimento principalmente na formação de centrossomos e mitose; formação de cílios primário e ciliopatias; e respostas a danos de DNA.
- O duplo híbrido em leveduras foi realizado utilizando-se o sistema Matchmaker GAL4 (Clontech Laboratories, Inc) no qual a isca foi a região C-terminal da proteína Nek5. No total 10 proteínas foram identificadas: MTX2, SNX6, DCAF10, C6orf225, CAP2, BCLAF1, SETDB1, TTLL4, TOP2B e COX11. A interação com algumas destas proteínas foram caracterizadas neste trabalho e outras ainda precisam ser exploradas.
- Uma vez que interagem com proteínas mitocôndrias a localização mitocondrial de Nek5 foi avaliada por microscopia confocal e fracionamento de mitocôndrias seguido por western blot, confirmando a presence de hNek5 neste microambiente.
- Ensaios de apoptose mostraram que células silenciadas para Nek5 apresentam maior sensibilidade ao tratamento com tapsigargina (2 μM), além de apresentarem níveis elevados de ROS.
- A atividade da cadeia respiratória mitocondrial estava diminuída após superexpressão de hNek5, especialmente nas etapas de transferência de elétrons do TMPD para o citocromo c e no complexo II.
- A interação de Nek5 com TTLL4 foi confirmada por imunoprecipitação e ensaios *in vitro* e *in vivo* demonstraram que na presença de Nek5 a atividade de TTLL4 é diminuída, e consequentemente a poliglutamilação de proteínas. Células silenciadas para a Nek5, assim como células que expressam a versão "kinase dead" de Nek5, apresentaram por *western blot* e ensaio *in vitro* de atividade poliglutamilação um aumento na poliglutamilação de proteínas após transfecção com TTLL4, o que não pode ser observado na presence de hNek5.

- Ensaios de imunoprecipitação seguido por espectrometria de massas permitiram a expansão do conhecimento sobre proteínas que interagem direta ou indiretamente com Nek5. No total 225 proteínas foram capazes de imunoprecipitar com Nek5. As proteínas identificadas estão envolvidas em processos biológicos como: expressão gênica, transcrição, tradução, apoptose e morte celular.
- A parceria entre o SGC e LNBIO permitiu a expressão em larga escala em bactéria das cinases Nek1, 6 e 7; além de ter resolvido a estrutura da Nek1 na presença e ausência de compostos inibitórios (PDB número 4B9D e 4APC respectivamente).

Referências Bibliográficas

Abal, M., Keryer, G., and Bornens, M. (2005). Centrioles resist forces applied on centrosomes during G2/M transition. Biol Cell, 97, 425-434.

Adams, J.M. and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. Science. 281(5381): 1322-6.

Ahmed, S.; Thomas, G.; Ghoussaini, M.; Healey, C. S.; Humphreys, M. K.; Platte, R.; Morrison, J.; Maranian, M.; Pooley, K. A.; Luben, R.; Eccles, D.; Evans, D. G.; Fletcher, O.; Johnson, N.; Dos Santos Silva, I.; Peto, J.; Stratton, M. R.; Rahman, N.; Jacobs, K.; Prentice, R.; Anderson, G. L.; Rajkovic, A.; Curb, J. D.; Ziegler, R. G.; Berg, C. D.; Buys, S. S.; Mccarty, C. A.; Feigelson, H. S.; Calle, E. E.; Thun, M. J.; et al. Newly discovered breast cancer susceptibility loci on 3p24 and 17q23.2. Nat Genet, v. 41, n. 5, p. 585-90, 2009.

Akhmanova, A., and Steinmetz, M.O. (2008). Tracking the ends: a dynamic protein network controls the fate of microtubule tips. Nat Rev Mol Cell Biol 9, 309-322.

Alexander, J.E., Hunt, D.F., Lee, M.K., Shabanowitz, J., Michel, H., Berlin, S.C., MacDonald, T.L., Sundberg, R.J., Rebhun, L.I. and Frankfurter, A. (1991) Characterization of posttranslational modifications in neuron-specific class III beta-tubulin by mass spectrometry. Proc Natl Acad Sci U S A, 88, 4685-4689.

Andreasson, U.; Dictor, M.; Jerkeman, M.; Berglund, M.; Sundstrom, C.; Linderoth, J.; Rosenquist, R.; Borrebaeck, C. A.; Ek, S. (2009). Identification of molecular targets associated with transformed diffuse large B cell lymphoma using highly purified tumor cells. Am J Hematol, v. 84, n. 12, p. 803-8.

Armstrong, LC.; Saenz, AJ and Bornstein, P. (1999). Metaxin 1 Interacts With Metaxin 2, a Novel Related Protein Associated With the Mammalian Mitochondrial Outer Membrane. Journal of Cellular Biochemistry.74:11–22. [PMID: 10381257].

Audebert, S., Desbruyeres, E., Gruszczynski, C., Koulakoff, A., Gros, F., Denoulet, P. and Eddé, B. (1993) Reversible polyglutamylation of alpha- and beta-tubulin and microtubule dynamics in mouse brain neurons. Mol Biol Cell, 4, 615-626.

Audebert, S., Koulakoff, A., Berwald-Netter, Y., Gros, F., Denoulet, P. and Eddé, B. (1994) Developmental regulation of polyglutamylated alpha- and beta-tubulin in mouse brain neurons. J Cell Sci, 107, 2313-2322.

Audebert, S., White, D., Cosson, J., Huitorel, P., Eddé, B. and Gagnon, C. (1999) The carboxy-terminal sequence Asp427-Glu432 of beta-tubulin plays an important function in axonemal motility. Eur J Biochem, 261, 48-56.

Backer, C.B.; Gutzman, J.H.; Pearson, C.G.; Cheeseman, I.M. (2012). CSAP localizes to polyglutamylated microtubules and promotes proper cilia function and zebrafish development. Mol Biol Cell. 2012 Jun;23(11):2122-30. doi: 10.1091/mbc.E11-11-0931. Epub 2012 Apr 4.

Banerjee, A. (2002). Coordination of posttranslational modifications of bovine brain alpha-tubulin. Polyglycylation of delta2 tubulin. J Biol Chem, 277, 46140-46144.

Barbagallo, F., Paronetto, M.P.; Franco, R.; Chieffi, P.; Dolci, S.; Fry, A.M. Geremia, R.; Sette, C. (2009). Increased expression and nuclear localization of the centrosomal kinase Nek2 in human testicular seminomas. J Pathol. 2009 Feb;217(3):431-41.

Barra, H.S., Arce, C.A., and Argarana, C.E. (1988). Posttranslational tyrosination/detyrosination of tubulin. Mol Neurobiol, 2, 133-153.

Belham, C.; Roig, J.; Caldwell, J.A.; Aoyama, Y.; Kemp, B.E.; Comb, M.; Avruch, J. (2003). A mitotic cascade of NIMA family kinases. Nercc1/Nek9 activates the Nek6 and Nek7 kinases. J Biol Chem. 12;278(37):34897-909. Epub 2003 Jul 2.

Benjamin, S., Weidberg, H.; Rapaport, D.; Pekar, O.; Nudelman, M.; Segal, D.; Hirschberg, K.; Katzav, S.; Ehrlich, M.; Horowitz, M (2011). EHD2 mediates trafficking from the plasma membrane by modulating Rac1 activity. Biochem J. 2011 Nov 1;439(3):433-42. doi: 10.1042/BJ20111010.

Benjamin, S.; Weidberg, H.; Rapaport, D.; Pekar, O.; Nudelman, M.; Segal, D.; Hirschberg, K.; Katzav, S.; Ehrlich, M.; Horowitz, M. EHD2 mediates trafficking from the plasma membrane by modulating Rac1 activity. Biochem J, v. 439, n. 3, p. 433-42, 2011.

Bergen, L.G.; Upshall, A., Morris, N.R. (1984) S-phase, G2, and nuclear division mutants of Aspergillus nidulans. J Bacteriol. 159: 114-119.Osmani et al., 1991

Berglund, L.; Bjorling, E.; Oksvold, P.; Fagerberg, L.; Asplund, A.; Szigyarto, C. A.; Persson, A.; Ottosson, J.; Wernerus, H.; Nilsson, P.; Lundberg, E.; Sivertsson, A.; Navani, S.; Wester, K.; Kampf, C.; Hober, S.; Ponten, F.; Uhlen, M. A genecentric Human

Protein Atlas for expression profiles based on antibodies. Mol Cell Proteomics, v. 7, n. 10, p. 2019-27, 2008.

Bertran, M.T.; Sdelci, S.; Regué, L.; Avruch, J.; Caelles, C, Roig J. (2011). Nek9 is a Plk1-activated kinase that controls early centrosome separation through Nek6/7 and Eg5. EMBO J. 2011 Jun 3;30(13):2634-47. doi: 10.1038/emboj.2011.179.

Bieling, P.; Kandels-Lewis, S.; Telley, I.A.; van Dijk, J.; Janke, C. and Surrey, T. (2008). CLIP-170 tracks growing microtubule ends by dynamically recognizing composite EB1/tubulin-binding sites. J Cell Biol 183, 1223-1233.

Bloom, L., Horvitz, H.R. (1997). The Caenorhabditis elegans gene unc-76 and its human homologs define a new gene family involved in axonal outgrowth and fasciculation. Proc Natl Acad Sci USA. 94:3414-9.

Blum, E.S.; Schwendeman, A.R.; Shaham, S. (2013). PolyQ disease: misfiring of a developmental cell death program?. Trends Cell Biol. 23(4):168-74.

Bobinnec, Y., Khodjakov, A., Mir, L.M., Rieder, C.L., Eddé, B. and Bornens, M. (1998a) Centriole disassembly in vivo and its effect on centrosome structure and function in vertebrate cells. J Cell Biol, 143, 1575-1589.

Bobinnec, Y., Moudjou, M., Fouquet, J.P., Desbruyeres, E., Eddé, B. and Bornens, M. (1998b) Glutamylation of centriole and cytoplasmic tubulin in proliferating non-neuronal cells. Cell Motil Cytoskeleton, 39, 223-232.

Bonnet, C., Boucher, D., Lazereg, S., Pedrotti, B., Islam, K., Denoulet, P. and Larcher, J.C. (2001) Differential Binding Regulation of Microtubule-associated Proteins MAP1A, MAP1B, and MAP2 by Tubulin Polyglutamylation. J Biol Chem, 276, 12839-12848.

Bosch Grau, M.; Gonzalez Curto, G; Rocha, C.; Magiera, M.M.; Marques Sousa, P.; Giordano, T.; Spassky, N.; Janke, C. (2013). Tubulin glycylases and glutamylases have distinct functions in stabilization and motility of ependymal cilia. J Cell Biol. 2013 Aug 5;202(3):441-51. doi: 10.1083/jcb.201305041. Epub 2013 Jul 29.

Boucher, D., Larcher, J.C., Gros, F. and Denoulet, P. (1994) Polyglutamylation of tubulin as a progressive regulator of in vitro interactions between the microtubule-associated protein Tau and tubulin. Biochemistry, 33, 12471-12477.

Bowers, A.J.; Boylan, J.F. (2004). Nek8, a NIMA family kinase member, is overexpressed in primary human breast tumors. Gene. 2004 Mar 17;328:135-42.

Bré, M. H., Redeker, V., Quibell, M., Darmanaden-Delorme, J., Bressac, C., Cosson, J., Huitorel, P., Schmitter, J. M., Rossler, J., Johnson, T., Adoutte, A., Levilliers, N. (1996) Axonemal tubulin polyglycylation probed with two monoclonal antibodies: widespread evolutionary distribution, appearance during spermatozoan maturation and possible function in motility. J Cell Sci, 109, 727-738.

Cao, X.; Xia, Y.; Yang, J.; Jiang, J.; Chen, L.; Ni, R.; Li, L.; Gu, Z. Clinical and biological significance of never in mitosis gene A-related kinase 6 (NEK6) expression in hepatic cell cancer. Pathol Oncol Res, v. 18, n. 2, p. 201-7, 2012.

Capra, M.; Nuciforo, P. G.; Confalonieri, S.; Quarto, M.; Bianchi, M.; Nebuloni, M.; Boldorini, R.; Pallotti, F.; Viale, G.; Gishizky, M. L.; Draetta, G. F.; Di Fiore, P. P. (2006). Frequent alterations in the expression of serine/threonine kinases in human cancers. Cancer Res, v. 66, n. 16, p. 8147-54.

Carazzolle, M.F.; de Carvalho, L.M.; Slepicka, H.H.; Vidal, R.O.; Pereira, G.A.; Kobarg, J.; Meirelles, G.V. (2014). IIS--Integrated Interactome System: a web-based platform for the annotation, analysis and visualization of protein-metabolite-gene-drug interactions by integrating a variety of data sources and tools. PLoS One. 2014 Jun 20;9(6):e100385. doi: 10.1371/journal.pone.0100385. eCollection 2014.

Carr, HS; George, GN and Winge, DR. 2002. Yeast Cox11, a protein essential for cytochrome c oxidase assembly, is a Cu(I)-binding protein. J. Biol. Chem. 277:31237–31242. [PMID: 12063264].

Carter, H.; Samayoa, J.; Hruban, R. H.; Karchin, R. Prioritization of driver mutations in pancreatic cancer using cancer-specific high-throughput annotation of somatic mutations (CHASM). Cancer Biol Ther, v. 10, n. 6, p. 582-7, 2010.

Chang, J.; Baloh, R.H.; Milbrandt, J. (2009). The NIMA-family kinase Nek3 regulates microtubule acetylation in neurons. J Cell Sci. 2009 Jul 1;122(Pt 13):2274-82. Epub 2009 Jun 9.

Chen, A.; Yanai, A.; Arama, E.; Kilfin, G.; Motro, B. (1999). NIMA-related kinases: isolation and characterization of murine nek3 and nek4 cDNAs, and chromosomal localization of nek1, nek2 and nek3. Gene. 1999 Jun 24;234(1):127-37.

Chen, J.; Li, L.; Zhang, Y.; Yang, H.; Wei, Y.; Zhang, L.; Liu, X.; Yu, L. (2006). Interaction of Pin1 with Nek6 and characterization of their expression. correlation in Chinese hepatocellular carcinoma patients. Biochem Biophys Res Commun. 2006 Mar 24;341(4):1059-65. Epub 2006 Jan 25.

Chen, Y.; Craigen, W.J.; Riley, D.J. (2009). Nek1 regulates cell death and mitochondrial membrane permeability through phosphorylation of VDAC1. Cell Cycle. 2009 Jan 15;8(2):257-67. Epub 2009 Jan 4.

Chen, Y.; Chen, C. F.; Riley, D. J.; Chen, P. L. (2011). Nek1 kinase functions in DNA damage response and checkpoint control through a pathway independent of ATM and ATR. Cell Cycle, v. 10, n. 4, p. 655 63.

Chen, Y.; Gaczynska, M.; Osmulski, P.; Polci, R.; Riley, D. J. (2010). Phosphorylation by Nek1 regulates opening and closing of voltage dependent anion channel 1. Biochem Biophys Res Commun, v. 394, n. 3, p. 798-803.

Chien, C.T.; Bartel, P.L.; Sternglanz, R.; Fields, S. (1991). The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc Natl Acad Sci U S A. 1991 Nov 1;88(21):9578-82.

Choi, H.J.; Lin, J.R.; Vannier, J.B.; Slaats, G.G.; Kile, A.C.; Paulsen, R.D.; Manning, D.K.; Beier, D.R.; Giles, R.H.; Boulton, S.J.; Cimprich, K.A. (2013). NEK8 links the ATR-regulated replication stress response and S phase CDK activity to renal ciliopathies. Mol Cell. 2013 Aug 22;51(4):423-39. doi: 10.1016/j.molcel.2013.08.006.

Cochran, S.D.; Cole, J.B.; Null, D.J.; Hansen, P.J. (2013). Single nucleotide polymorphisms in candidate genes associated with fertilizing ability of sperm and subsequent embryonic development in cattle.

Coene, K. L.; Mans, D. A.; Boldt, K.; Gloeckner, C. J.; Van Reeuwijk, J.; Bolat, E.; Roosing, S.; Letteboer, S. J.; Peters, T. A.; Cremers, F. P.; Ueffing, M.; Roepman, R. (2011). The ciliopathy-associated protein homologs RPGRIP1 and RPGRIP1L are linked to cilium integrity through interaction with Nek4 serine/threonine kinase. Hum Mol Genet, v. 20, n. 18, p. 3592-605.

Cohen, S.; Aizer, A.; Shav-Tal, Y.; Yanai, A.; Motro, B. (2013). Nek7 kinase accelerates microtubule dynamic instability. Biochim Biophys Acta. 2013 May;1833(5):1104-13. doi:10.1016/j.bbamcr.2012.12.021. Epub 2013 Jan 8.

Cory, S. and Adams, J.M. (2002). The Bcl-2 family: regulators of cell life or death sqitch. Nat Rev Cancer. 2(9):647-56.

Cosson, J., White, D., Huitorel, P., Eddé, B., Cibert, C., Audebert, S. and Gagnon, C. (1996) Inhibition of flagellar beat frequency by a new anti-beta-tubulin antibody. Cell Motil Cytoskeleton, 35, 100-112.

Das, V.; Kanakkanthara, A.; Chan, A.; Miller, J.H. (2014). Potential role of tubulin tyrosine ligase-like enzymes in tumorigenesis and cancer cell resistance. Cancer Lett. 2014 Aug 1;350(1-2):1-4. doi: 10.1016/j.canlet.2014.04.022. Epub 2014 May 6. Review.

De Vos, S.; Hofmann, W. K.; Grogan, T. M.; Krug, U.; Schrage, M.; Miller, T. P.; Braun, J. G.; Wachsman, W.; Koeffler, H. P.; Said, J. W. Gene expression profile of serial samples of transformed B-cell lymphomas. Lab Invest, v. 83, n. 2, p. 271-85, 2003.

Deeks, M.J.; Rodrigues, C.; Dimmock, S.; Ketelaar, T.; Maciver, S.K.; Malhó, R. Hussey, P.J. (2007). Arabidopsis CAP1 - a key regulator of actin organisation and development. J Cell Sci. 2007 Aug 1;120(Pt 15):2609-18. Epub 2007 Jul 17.

Doles, J.; Hemann, M. T. (2010). Nek4 status differentially alters sensitivity to distinct microtubule poisons. Cancer Res, v. 70, n. 3, p. 1033-41, 2010.

Drozdov, I.; Bornschein, J.; Wex, T.; Valeyev, N.V.; Tsoka, S.; Malfertheiner, P. (2012). Functional and topological properties in hepatocellular carcinoma transcriptome. PLoS One. 2012;7(4):e35510. doi: 10.1371/journal.pone.0035510. Epub 2012 Apr 23.

Dunham, A., Matthews, L.H., Burton, J., Ashurst, J.L., Howe, K.L., Ashcroft, K.J., Beare, D.M., Burford, D.C., Hunt, S.E., Griffiths-Jones, S., Jones, M.C., Keenan, S.J., Oliver, K., Scott, C.E., Ainscough, R., Almeida, J.P., Ambrose K.D., Andrews D.T. Ross M.T. (2004). The DNA sequence and analysis of human chromosome 13. Nature 428:522-528.

Eddé, B.; Rossier, J.; Le Caer, J.P.; Berwald-Netter, Y.; Koulakoff, A.; Gros, F. and Denoulet, P. (1991) A combination of posttranslational modifications is responsible for the production of neuronal alpha-tubulin heterogeneity. J Cell Biochem, 46, 134-142.

Eddé, B.,; Rossier, J.; Le Caer, J.P.; Desbruyeres, E.; Gros, F. and Denoulet, P. (1990) Posttranslational glutamylation of alpha-tubulin. Science, 247, 83-85.

Eddé, B.; Rossier, J.; Le Caer, J.P.; Prome, J.C.; Desbruyeres, E.; Gros, F. and Denoulet, P. (1992) Polyglutamylated alpha-tubulin can enter the tyrosination/detyrosination cycle. Biochemistry, 31, 403-410.

Elmore, S. 2007. Apoptosis: a review of programmed cell death. Toxicol Pathol. 35(4): 496-516.

Ersfeld, K.; Wehland, J.; Plessmann, U.; Dodemont, H.; Gerke, V. and Weber, K. (1993). Characterization of the tubulin-tyrosine ligase. J Cell Biol 120, 725-732.

Fields, S.; Song, O. (1989). A novel genetic system to detect protein-protein interactions. Nature. 1989 Jul 20;340(6230):245-6.

Fields, S.; Sternglanz, R. (1994). The two-hybrid system: an assay for protein-protein interactions. Trends Genet. 10:286-292.

Fletcher, L.; Cerniglia, G. J.; Yen, T. J.; Muschel, R. J. (2005). Live cell imaging reveals distinct roles in cell cycle regulation for Nek2A and Nek2B. Biochim. Biophys. Acta.1744(2): 89–92.

Forrest, A.R.; Taylor, D.; Grimmond, S. (2003) Exploration of the cell-cycle genes found within the RIKEN FANTOM2 data set. Genome Res. 13(6B):1366-1375.

Fouquet, J.P., Eddé, B., Kann, M.L., Wolff, A., Desbruyeres, E. and Denoulet, P. (1994) Differential distribution of glutamylated tubulin during spermatogenesis in mammalian testis. Cell Motil Cytoskeleton, 27, 49-58.

Frank, V.; Habbig, S.; Bartram, M.P.; Eisenberger, T.; Veenstra-Knol, H.E.; Decker, C.; Boorsma, R.A.; Göbel, H.; Nürnberg, G.; Griessmann, A.; Franke, M.; Borgal, L.; Kohli, P.; Völker, L.A.; Dötsch, J.; Nürnberg, P.; Benzing, T.; Bolz, H.J.; Johnson, C.; Gerkes, E.H.; Schermer, B.; Bergmann, C. (2013). Mutations in NEK8 link multiple organ dysplasia with altered Hippo signalling and increased c-MYC expression. Hum Mol Genet. 2013 Jun 1;22(11):2177-85. doi: 10.1093/hmg/ddt070. Epub 2013 Feb 14.

Fry, A.M.; Descombes, P.; Twomey, C.; Bacchieri, R.; Nigg, E.A. (2000). The NIMA-related kinase X-Nek2B is required for efficient assembly of the zygotic centrosome in Xenopus laevis. J Cell Sci. 2000 Jun;113 (Pt 11):1973-84.

Fry, A.M.; Meraldi P, Nigg EA. A centrosomal function for the human NEK2 protein kinase, a member of the NIMA family of cell cycle regulators. EMBO J. 1998, 17, 470-481.

Fry, A.M. O'Regan, L.; Sabir, S.R.; Bayliss, R. (2012). Cell cycle regulation by the NEK family of protein kinases. J Cell Sci. 2012 Oct 1;125(Pt 19):4423-33. doi: 10.1242/jcs.111195. Epub 2012 Nov 6. Review.

Fry, A. M.; Arnaud, L.; Nigg, E. A. (1999). Activity of the human centrosomal kinase, Nek2, depends on an unusual leucine zipper dimerization motif. J Biol Chem, v. 274, n. 23, p. 16304-10.

Fry, A. M.; Schultz, S. J.; Bartek, J.; Nigg, E. A. (1995). Substrate specificity and cell cycle regulation of the Nek2 protein kinase, a potential human homolog of the mitotic regulator NIMA of Aspergillus nidulans. J Biol Chem, v. 270, n. 21, p. 12899-905, 1995.

Fry, A.M. The Nek2 protein kinase: a novel regulator of centrosome structure. Oncogene, v. 21, n. 40, p. 6184-94, 2002.

Fujita, T.; Maturana, A.D.; Ikuta, J.; Hamada, J.; Walchli, S.; Suzuki, T.; Sawa, H.; Wooten, M.W.; Okajima, T.; Tatematsu, K.; Tanizawa, K.; Kuroda, S. (2007). Axonal guidance protein FEZ1 associates with tubulin and kinesin motor protein to transport mitochondria in neurites of NGF-stimulated PC12 cells. Biochem Biophys Res Commun. 361:605-10.

Fukui, H.; Shiba, D.; Asakawa, K.; Kawakami, K.; Yokoyama, T. (2012). The ciliary protein Nek8/Nphp9 acts downstream of Inv/Nphp2 during pronephros morphogenesis and left-right establishment in zebrafish. FEBS Lett. 2012 Jul 30;586(16):2273-9. doi: 10.1016/j.febslet.2012.05.064. Epub 2012 Jun 8.

Fullston, T.; Gabb, B.; Callen, D.; Ullmann, R.; Woollatt, E.; Bain, S.; Ropers, H.H.; Cooper, M.; Chandler, D.; Carter, K.; Jablensky, A.; Kalaydjieva, L.; Gecz, J. (2011). Inherited balanced translocation t(9;17)(q33.2;q25.3) concomitant with a 16p13.1 duplication in a patient with schizophrenia. Am J Med Genet B Neuropsychiatr Genet. 2011 Mar;156(2):204-14. doi: 10.1002/ajmg.b.31157. Epub 2011 Jan 13.

Gagnon, C., White, D., Cosson, J., Huitorel, P., Eddé, B., Desbruyeres, E., Paturle-Lafanechere, L., Multigner, L., Job, D. and Cibert, C. (1996) The polyglutamylated lateral chain of alpha-tubulin plays a key role in flagellar motility. J Cell Sci, 109, 1545-1553.

Gentry, M. S., and Hallberg, R. L. (2002) Mol. Biol. Cell. 13, 3477.

Gindhart, J.G.; Chen, J.; Faulkner, M.; Gandhi, R.; Doerner, K.; Wisniewski, T.; Nandlestadt, A. (2003) The kinesin-associated protein UNC-76 is required for axonal transport in the Drosophila nervous system. Mol Biol Cell 14:3356-65.

Greenman, C.; Stephens, P.; Smith, R.; Dalgliesh, G.L.; Hunter, C.; Bignell, G.; Davies, H.; Teague, J.; Butler, A.; Stevens, C.. (2007) Patterns of somatic mutation in human cancer genomes. Nature. 446(7132): 153-158.

Grusche, F.A.; Hidalgo, C.; Fletcher, G.; Sung, H.H.; Sahai, E.; Thompson, B.J. (2009). Sds22, a PP1 phosphatase regulatory subunit, regulates epithelial cell polarity and shape [Sds22 in epithelial morphology]. BMC Dev Biol. 19;9:14.

Guido, Sauter, Ronald Simon & Kenneth Hillan. (2003). Tissue microarrays in drug discovery. Nature Reviews Drug Discovery. 2: 962-972.

Habbig, S.; Bartram, M.P.; Sägmüller, J.G.; Griessmann, A.; Franke, M.; Müller, R.U.; Schwarz, R.; Hoehne, M.; Bergmann, C.; Tessmer, C.; Reinhardt, H.C.; Burst, V.; Benzing, T; Schermer, B. (2012). The ciliopathy disease protein NPHP9 promotes nuclear delivery and activation of the oncogenic transcriptional regulator TAZ. Hum Mol Genet. 2012 Dec 15;21(26):5528-38. doi: 10.1093/hmg/dds408. Epub 2012 Oct 1.

Hames, R.S.; Fry, A.M. (2002). Alternative splice variants of the human centrosome kinase Nek2 exhibit distinct patterns of expression in mitosis. Biochem J. 2002 Jan 1;361(Pt 1):77-85.

Hashimoto, Y.; Akita, H.; Hibino, M.; Kohri, K.; Nakanishi, M. (2002). Identification and characterization of Nek6 protein kinase, a potential human homolog of NIMA histone H3 kinase. Biochem Biophys Res Commun. 2002 May 3;293(2):753-8.

Okada, H.; Zhang, W.; Peterhoff, C.; Hwang, J.C,; Nixon, R.A.; Ryu, S.H. and Kim, T.W.. (2010). Proteomic identification of sorting nexin 6 as a.

Holland, P.M.; Milne, A.; Garka, K.; Johnson, R.S.; Willis, C.; Sims, J.E.; Rauch, C.T.; Bird, T.A.; Virca, G.D. (2002). Purification, cloning, and characterization of Nek8, a novel NIMA-related kinase, and its candidate substrate Bicd2. J Biol Chem. 2002 May 3;277(18):16229-40. Epub 2002 Feb 25.

Holloway, K.; Roberson, E. C.; Corbett, K. L.; Kolas, N. K.; Nieves, E.; Cohen, P. E. (2011). NEK1 Facilitates Cohesin Removal during Mammalian Spermatogenesis. Genes (Basel), v. 2, n. 1, p. 260-279, 2011.

Honda, A.; Miyoshi, K; Baba, K.; Taniguchi, M.; Koyama, Y.; Kuroda, S.; Katayama, T.; Tohyama, M.(2004) Expression of fasciculation and elongation protein zeta-1 (FEZ1) in the developing rat brain. Brain Res Mol Brain Res 122:89-92.

Horvitz, H.R. (1999). Genetic control of programmed cell death in the nematode Caenorhabditis elegans. Cancer Res. 59(7):1701-1706.

Huang, L.Y.; Chang, C.C.; Lee, Y.S.; Huang, J.J.; Chuang, S.H.; Chang, J.M.; Kao, K.J.; Lau, G.M.; Tsai, P.Y.; Liu, C.W.; Lin, H.S.; Gish, R.G.; Lau, J.Y.

Huang, L.Y.; Lee, Y.S.; Huang, J.J.; Chang, C.C.; Chang, J.M.; Chuang, S.H.; Kao, K.J.; Tsai, Y.J.; Tsai, P.Y.; Liu, C.W.; Lin, H.S.; Lau, J.Y. (2014). Characterization of the biological activity of a potent small molecule Hec1 inhibitor TAI-1.

Iftode, F.; Clerot, J.C.; Levilliers, N.; and Bré, M.H. (2000). Tubulin polyglycylation: a morphogenetic marker in ciliates. Biol Cell, 92, 615-628.

Ikegami K; Horigome D; Mukai M; Livnat I; MacGregor GR; Setou M. (2008). TTLL10 is a protein polyglycylase that can modify nucleosome assembly protein 1. FEBS Lett. 2008 Apr 2;582(7):1129-34. doi: 10.1016/j.febslet.2008.02.079. Epub 2008 Mar 10.

Ikegami K; Setou M. (2009). TTLL10 can perform tubulin glycylation when co-expressed with TTLL8. FEBS Lett. 2009 Jun 18;583(12):1957-63. doi: 10.1016/j.febslet.2009.05.003. Epub 2009 May 8.

Ikegami K; Setou M. (2010). Unique post-translational modifications in specialized microtubule architecture. Cell Struct Funct. 2010;35(1):15-22. Epub 2010 Feb 27.

Ikegami, K.; Heier, R.L.; Taruishi, M.; Takagi, H.; Mukai, M.; Shimma, S.; Taira, S.; Hatanaka, K.; Morone, N.; Yao, I.; Campbell, P.K.; Yuasa, S.; Janke, C.; MacGregor, G.R. and Setou; M. (2007) Loss of alpha-tubulin polyglutamylation in ROSA22 mice is associated with abnormal targeting of KIF1A and modulated synaptic function. Proc Natl Acad Sci U S A, 104, 3213–3218.

Ikegami, K.; Mukai, M.; Tsuchida, J.-i.; Heier, R.L.; Macgregor, G.R. and Setou; M. (2006) TTLL7 is a mammalian beta-tubulin polyglutamylase required for growth of MAP2-positive neurites. J Biol Chem, 281, 30707-30716.

Ikuta J; Maturana A; Fujita T; Okajima T; Tatematsu K; Tanizawa K; Kuroda S. (2007). Fasciculation and elongation protein zeta-1 (FEZ1) participates in the polarization

of hippocampal neuron by controlling the mitochondrial motility. Biochem Biophys Res Commun 353:127-32.

Jackson PK. (2013). Nek8 couples renal ciliopathies to DNA damage and checkpoint control. Mol Cell. 2013 Aug 22;51(4):407-8. doi: 10.1016/j.molcel.2013.08.013.

Janke, C.; Rogowski, K.; and van Dijk, J. (2008). Polyglutamylation: a fine-regulator of protein function? 'Protein Modifications: beyond the usual suspects' review series. EMBO Rep 9, 636-641.

Janke, C.; Rogowski, K.; Wloga, D.; Regnard, C.; Kajava, A.; Strub, J.-M.; Temurak, N.; Van Dijk, J.; Boucher, D.; Van Dorsselaer, A.; et al. (2005). Tubulin Polyglutamylase Enzymes are Members of the TTL Domain Protein Family. Science, 308, 1758-1762.

Jee HJ; Kim HJ; Kim AJ; Song N; Kim M; Lee HJ; Yun J.(2013). The inhibition of Nek6 function sensitizes human cancer cells to premature senescence upon serum reduction or anticancer drug treatment. Cancer Lett. 2013 Jul 10;335(1):175-82. doi: 10.1016/j.canlet.2013.02.012. Epub 2013 Feb 13.

Jee HJ; Kim HJ; Kim AJ; Song N; Kim M; Yun J. (2011). Nek6 suppresses the premature senescence of human cancer cells induced by camptothecin and doxorubicin treatment. Biochem Biophys Res Commun. 2011 May 20;408(4):669-73. Epub 2011 Apr 23.

Jeffery JM; Urquhart AJ; Subramaniam VN; Parton RG; Khanna KK. (2010). Centrobin regulates the assembly of functional mitotic spindles. Oncogene. 2010 May 6;29(18):2649-58. Epub 2010 Mar 1.

Jeong AL; Lee S; Park JS; Han S; Jang CY; Lim JS; Lee MS; Yang Y.Kerr. (2013). Cancerous inhibitor of protein phosphatase 2A (CIP2A) protein is involved in centrosome separation through the regulation of NIMA (never in mitosis gene A)-related kinase 2 (NEK2) protein activity. J Biol Chem. 2014 Jan 3;289(1):28-40. doi: 10.1074/jbc.M113.507954. Epub 2013 Nov 8.

Kalinina, E.; R. Biswas, I. Berezniuk; A. Hermoso; F.X. Aviles; and L.D. Fricker. 2007. A novel subfamily of mouse cytosolic carboxypeptidases. Faseb J. 21:836-850.

Kandli M; Feige E; Chen A; Kilfin G; Motro B. (2000). Isolation and characterization of two evolutionarily conserved murine kinases (Nek6 and nek7) related to the fungal mitotic regulator, NIMA. Genomics. 2000 Sep 1;68(2):187-96.

Kaneta Y; Ullrich A. (2013). NEK9 depletion induces catastrophic mitosis by impairment of mitotic checkpoint control and spindle dynamics. Biochem Biophys Res Commun. 2013 Dec 13;442(3-4):139-46. doi: 10.1016/j.bbrc.2013.04.105. Epub 2013 May 9.

Kasap, E.; Boyacioglu, S. O.; Korkmaz, M.; Yuksel, E. S.; Unsal, B.; Kahraman, E.; Ozutemiz, O.; Yuceyar, H. Aurora kinase A (AURKA) and never in mitosis gene A-related kinase 6 (NEK6) genes are upregulated in erosive esophagitis and esophageal adenocarcinoma. Exp Ther Med, v. 4, n. 1, p. 33-42, 2012.

Kashiwaya, K.; Nakagawa, H.; Hosokawa, M.; Mochizuki, Y.; Ueda, K.; Piao, L.; Chung, S.; Hamamoto, R.; Eguchi, H.; Ohigashi, H.; Ishikawa, O.; Janke, C.; Shinomura, Y.; Nakamura, Y. (2010). Involvement of the tubulin tyrosine ligase-like family member 4 polyglutamylase in PELP1 polyglutamylation and chromatin remodeling in pancreatic cancer cells, Cancer Res. 70: 4024–4033.

Kasof, GM; Goyal, L and White, E. (1999). Btf, a novel death-promoting transcriptional repressor that interacts with Bcl-2-related proteins. Mol. Cell. Biol. 19 (6), 4390–404. [PMID: 10330179].

Ken Yamazaki; Masaaki Takamura2; Yohei Masugi1; Taisuke Mori1; Wenlin Du1; Taizo Hibi3; Nobuyoshi Hiraoka4; Tsutomu Ohta4; Misao Ohki4; Setsuo Hirohashi5 and Michiie Sakamoto1. (2009). Laboratory Investigation (2009) 89, 425–432.

Kim S; Kim S; Rhee K. (2012). NEK7 is essential for centriole duplication and centrosomal accumulation of pericentriolar material proteins in interphase cells. J Cell Sci. 2011 Nov 15;124(Pt 22):3760-70. Epub 2011 Nov 18.

Kim S; Lee K; Rhee K. (2007). NEK7 is a centrosomal kinase critical for microtubule nucleation. Biochem Biophys Res Commun. 2007 Aug 17;360(1):56-62. Epub 2007 Jun 8.

Kim; S.; Rhee; K. (2011). NEK7 is essential for centriole duplication and centrosomal accumulation of pericentriolar material proteins in interphase cells. J Cell Sci, v. 124, n. Pt 22, p. 3760-70.

Kimura M; Okano Y (2001) Molecular cloning and characterization of the human NIMA-related protein kinase 3 gene (NEK3). Cytogenet Cell Genet. 2001;95(3-4):177-82.

Koch M; Wiese M. (2013). Gene expression signatures of angiocidin and darapladib treatment connect to therapy options in cervical cancer. J Cancer Res Clin Oncol. 2013 Feb;139(2):259-67. doi: 10.1007/s00432-012-1317-9. Epub 2012 Oct 9.

Kohler, M. E.; Johnson, B. D.; Palen, K.; Chen, Q. R.; Khan, J.; Orentas, R. J. (2010). Tumor antigen analysis in neuroblastoma by serological interrogation of bioinformatic data. Cancer Sci, v. 101, n. 11, p. 2316-24, 2010.

Kokuryo, T.; Senga, T.; Yokoyama, Y.; Nagino, M.; Nimura, Y.; Hamaguchi, M. Nek2 as an effective target for inhibition of tumorigenic growth and peritoneal dissemination of cholangiocarcinoma. Cancer Res, v. 67, n. 20, p. 9637-42, 2007.

Kozjak-Pavlovic, V; Ross, K; Benlasfer, N; Kimmig, S; Karlas, A; Rudel, T. (2007). Conserved roles of Sam50 and metaxins in VDAC biogenesis. EMBO Rep. 2007 Jun;8(6):576-82. [PMID: 17510655].

Lachke SA; Higgins AW; Inagaki M; Saadi I; Xi Q; Long M; Quade BJ; Talkowski ME; Gusella JF; Fujimoto A; Robinson ML; Yang Y; Duong QT; Shapira I; Motro B; Miyoshi J; Takai Y; Morton CC; Maas RL. (2012). The cell adhesion gene PVRL3 is associated with congenital ocular defects. Hum Genet. 2012 Feb;131(2):235-50. doi: 10.1007/s00439-011-1064-z. Epub 2011 Jul 17.

Lafanechere, L.; and Job, D. (2000). The third tubulin pool. Neurochem Res, 25, 11-18.

Landi, M. T.; Dracheva, T.; Rotunno, M.; Figueroa, J. D.; Liu, H.; Dasgupta, A.; Mann, F. E.; Fukuoka, J.; Hames, M.; Bergen, A. W.; Murphy, S. E.; Yang, P.; Pesatori, A. C.; Consonni, D.; Bertazzi, P. A.; Wacholder, S.; Shih, J. H.; Caporaso, N. E.; Jen, J. Gene expression signature of cigarette smoking and its role in lung adenocarcinoma development and survival. PLoS One, v. 3, n. 2, p. e1651, 2008.

Lanza DC; Meirelles GV; Alborghetti MR; Abrile CH; Lenz G; Kobarg J. (2010). FEZ1 interacts with CLASP2 and NEK1 through coiled-coil regions and their cellular colocalization suggests centrosomal functions and regulation by PKC. Mol Cell Biochem. 338(1-2):35-45.

Lapenna, S.; Giordano, A. Cell cycle kinases as therapeutic targets for cancer. Nat Rev Drug Discov, v. 8, n.7, p. 547-66, 2009.

Larcher, J.C.; Boucher, D.; Lazereg, S.; Gros, F. and Denoulet, P. (1996) Interaction of kinesin motor domains with alpha- and beta-tubulin subunits at a tau-independent binding site. Regulation by polyglutamylation. J Biol Chem, 271, 22117-22124.

Lechtreck, K.F.; and Geimer, S. (2000). Distribution of polyglutamylated tubulin in the flagellar apparatus of green flagellates. Cell Motil Cytoskeleton, 47, 219-235.

Lee J; Gollahon L. (2013). Nek2-targeted ASO or siRNA pretreatment enhances anticancer drug sensitivity in triple□negative breast cancer cells. Int J Oncol. 2013 Mar;42(3):839-47. doi: 10.3892/ijo.2013.1788. Epub 2013 Jan 22.

Lee MY; Kim HJ; Kim MA; Jee HJ; Kim AJ; Bae YS; Park JI; Chung JH; Yun J. (2008). Nek6 is involved in G2/M phase cell cycle arrest through DNA damage-induced phosphorylation. Cell Cycle. 1;7(17):2705-9. Epub 2008 Sep 3.

Lee, YY; Yu, YB; Gunawardena, HP; Xie, L and Chen, X. (2012). BCLAF1 is a radiation-induced H2AX-interacting partner involved in cH2AX-mediated regulation of apoptosis and DNA repair. Cell Death Dis. 26;3:e359. [PMID: 22833098].

Letwin K; Mizzen L; Motro B; Ben-David Y; Bernstein A; Pawson T. A mammalian dual specificity protein kinase, Nek1, is related to the NIMA cell cycle regulator and highly expressed in meiotic germ cells. EMBO J 1992; 11: 3521-3531 [PMID: 1382974].

Lewandowski NM; Bordelon Y; Brickman AM; Angulo S; Khan U; Muraskin J; Griffith EY; Wasserman P; Menalled L; Vonsattel JP; Marder K; Small SA; Moreno H. (2013). Regional vulnerability in Huntington's disease: fMRI-guided molecular analysis in patients and a mouse model of disease. Neurobiol Dis. 52:84-93.

Lexander, H.; Palmberg, C.; Hellman, U.; Auer, G.; Hellström, M.; Franzén, B.; Jörnvall, H.; Egevad, L. (2006). Correlation of protein expression, Gleason score and DNA ploidy in prostate cancer. Proteomics 2006, 6, 4370–4380.

Liang H; Cheung LW; Li J; Ju Z; Yu S; Stemke-Hale K; Dogruluk T; Lu Y; Liu X; Gu C; Guo W; Scherer SE; Carter H; Westin SN; Dyer MD; Verhaak RG; Zhang F; Karchin R; Liu CG; Lu KH; Broaddus RR; Scott KL; Hennessy BT; Mills GB. (2012). Whole-exome sequencing combined with functional genomics reveals novel candidate

driver cancer genes in endometrial cancer. Genome Res. 2012 Nov;22(11):2120-9. doi: 10.1101/gr.137596.112. Epub 2012 Oct 1.

Link, C.D. and Saldi, T.K. (2012) Cell Death by Glutamine Repeats? Science 335, 926 (2012).

Liu S; Lu W; Obara T; Kuida S; Lehoczky J; Dewar k; Drummond IA, Beier DA. (2002) A defect in a novel Nek-family kinase causes cystic kidney disease in the mouse and in zebrafish. Development and Disease. 129(24): 5839-5846.

Liu X; Gao Y; Lu Y; Zhang J; Li L; Yin F. (2014). Upregulation of NEK2 is associated with drug resistance in ovarian cancer. Oncol Rep. 2014 Feb;31(2):745-54. doi: 10.3892/or.2013.2910. Epub 2013 Dec 9.

Liu, Q.; Hirohashi, Y.; Du, X.; Greene, M. I.; Wang, Q. (2010). Nek2 targets the mitotic checkpoint proteins Mad2 and Cdc20: a mechanism for aneuploidy in cancer. Exp Mol Pathol, v. 88, n. 2, p. 225-33.

Liu, S.; Ho, C. K.; Ouyang, J.; Zou, L. (2013). Nek1 kinase associates with ATR-ATRIP and primes ATR for efficient DNA damage signaling. Proc Natl Acad Sci U S A, v. 110, n. 6, p. 2175-80.

Löffler, H.; Rebacz, B.; Ho, A.D.; Lukas, J.; Bartek, J.; Krämer, A. (2006). Chk1-dependent regulation of Cdc25B functions to coordinate mitotic events. Cell Cycle. 1;5(21):2543-7

Lou Y; Yao J; Zereshki A; Dou Z; Ahmed K; Wang H; Hu J; Wang Y; Yao X. (2004). NEK2A interacts with MAD1 and possibly functions as a novel integrator of the spindle checkpoint signaling. J Biol Chem. 2004 May 7;279(19):20049-57. Epub 2004 Feb 20.

Lu KP; Hunter T. (1995) Evidence for a NIMA-like mitotic pathway in vertebrate cells. Cell. 81: 413-24.

Luduena, R.F. (1998). Multiple forms of tubulin: different gene products and covalent modifications. Int Rev Cytology, 178, 207-275.

M. D. Michelitsch, J. S. Weissman, Proc. Natl. Acad. Sci.U.S.A. 97, 11910 (2000).

Mahjoub MR; Trapp ML; Quarmby LM: (2005). NIMA-related kinases defective in murine models of polycystic kidney diseases localize to primary cilia and centrosomes. J Am Soc Nephrol 2005, 16:3485-3489.

Malumbres, M.; Barbacid, M. Cell cycle kinases in cancer. Curr Opin Genet Dev, v. 17, n. 1, p. 60-5, 2007.

Manevich, Y.; Fisher, A.B. (2005). Peroxiredoxin 6, a 1-Cys peroxiredoxin, functions in antioxidant defense and lung phospholipid metabolism. Free Radic Biol Med 2005, 38:1422-1432.

Manning G1; Whyte DB; Martinez R; Hunter T; Sudarsanam S. (2002) The protein kinase complement of the human genome. Science. 298(5600): 1912-1934.

Mardin, B. R.; Lange, C.; Baxter, J. E.; Hardy, T.; Scholz, S. R.; Fry, A. M.; Schiebel, E. (2010). Components of the Hippo pathway cooperate with Nek2 kinase to regulate centrosome disjunction. Nat Cell Biol, v. 12, n. 12, p. 1166-76.

Marina, M.; Saavedra, H. I. Nek2 and Plk4: prognostic markers, drivers of breast tumorigenesis and drug resistance. Front Biosci (Landmark Ed), v. 19, p. 352-65, 2014.

Martinou JC; Youle RJ (2011) Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. Dev. Cell 21(1), 92–101.

Mary, J.; Redeker, V.; Le Caer, J.P.; Prome, J.C. and Rossier, J. (1994) Class I and IVa beta-tubulin isotypes expressed in adult mouse brain are glutamylated. FEBS Lett, 353, 89-94.

Massoner, P.; Lueking, A.; Goehler, H.; Höpfner, A.; Kowald, A.; Kugler, K.G.; Amersdorfer, P.; Horninger, W.; Bartsch, G.; Schulz-Knappe, P.; Klocker, H. (2012). Serumautoantibodies for discovery of prostate cancer specific biomarkers. Prostate. 72: 427–436.

Matsuo, K.; Nishimura, T.; Hayakawa, A.; Ono, Y.; Takahashi, M. (2010). Involvement of a centrosomal protein kendrin in the maintenance of centrosome cohesion by modulating Nek2A kinase activity. Biochem Biophys Res Commun, v. 398, n. 2, p. 217-23, 2010.

Mazumder, S; Plesca, D and Almasan, A. (2007). A jekyll and hyde role of cyclin E in the genotoxic stress response: switching from cell cycle control to apoptosis regulation. Cell Cycle 6: 1437–1442. [PMID: 17581275].Zeviani et al., 1998.

Mchale, K.; Tomaszewski, J. E.; Puthiyaveettil, R.; Livolsi, V. A.; Clevenger, C. V. Altered expression of prolactin receptor-associated signaling proteins in human breast carcinoma. Mod Pathol, v. 21, n. 5, p. 565- 71, 2008.

McPherson, J.P.; Sarras, H.; Lemmers, B.; Migon, E.; Matysiak-Zablocki, E.; Hakem, A.; Calarco, J.A.; Alizadeh Azami, S.; Cardoso, R.; Tamblyn, L.; Fish, J.; Sanchez, O.; Post, M.; and Hakem, R. (2009) Essential role for Bcl-2 associated factor 1 in lung development and immune system function. Cell Death Differ. 16, 331–339.

Melixetian, M.; Klein, D. K.; Sorensen, C. S.; Helin, K. NEK11 regulates CDC25A degradation and the IRinduced G2/M checkpoint. Nat Cell Biol, v. 11, n. 10, p. 1247-53, 2009.

Miller SL; DeMaria JE; Freier DO; Riegel AM; Clevenger CV. (2005). Novel association of Vav2 and Nek3 modulates signaling through the human prolactin receptor. Mol Endocrinol. 2005 Apr;19(4):939-49. Epub 2004 Dec 23.

Miller SL; Antico G; Raghunath PN; Tomaszewski JE; Clevenger CV. (2007) Nek3 kinase regulates prolactin-mediated cytoskeletal reorganization and motility of breast cancer cells. Oncogene. 2007 Jul 12;26(32):4668-78. Epub 2007 Feb 12.

Million, K.; Larcher, J.; Laoukili, J.; Bourguignon, D.; Marano, F. and Tournier, F. (1999) Polyglutamylation and polyglycylation of alpha- and beta-tubulins during in vitro ciliated cell differentiation of human respiratory epithelial cells. J Cell Sci, 112, 4357-4366.

Miyoshi K; Kasahara K; Miyazaki I; Asanuma M. (2011) Factors that influence primary cilium length. Acta Med Okayama. 65(5): 279-285.

Moniz, L. S.; Stambolic, V. Nek10 mediates G2/M cell cycle arrest and MEK autoactivation in response to UV irradiation. Mol Cell Biol, v. 31, n. 1, p. 30-42, 2011.

Mori T; Nishizawa S; Hirohashi Y; Torigoe T; Tamura Y; Takahashi A; Kochin V; Fujii R; Kondo T; Greene MI; Hara I; Sato N. 2012. Efficiency of G2/M-related tumor-associated antigen-targeting cancer immunotherapy depends on antigen expression in the cancer stem-like population. Exp Mol Pathol. 2012 Feb;92(1):27-32. Epub 2011 Oct 2.

Nassirpour R; Shao L; Flanagan P; Abrams T; Jallal B; Smeal T; Yin MJ. (2010) Nek6 Mediates Human Cancer Cell Transformation and Is a Potential Cancer Therapeutic Target Mol Cancer Res. 8(5): 717-28.

Nguyen CL; Possemato R; Bauerlein EL; Xie A; Scully R; Hahn WC. (2012). Nek4 regulates entry into replicative senescence and the response to DNA damage in human fibroblasts. Mol Cell Biol. 2012 Oct;32(19):3963-77. doi: 10.1128/MCB.00436-12. Epub 2012 Jul 30.

Nogales; E. (2000). Structural insights into microtubule function. Annu Rev Biochem, 69, 277-302.

Noguchi, K.; Fukazawa, H.; Murakami, Y.; Uehara, Y. (2002). Nek11, a new member of the NIMA family of kinases, involved in DNA replication and genotoxic stress responses. J Biol Chem, v. 277, n. 42, p. 39655-65.

Noguchi, K.; Fukazawa, H.; Murakami, Y.; Uehara, Y. Nucleolar Nek11 is a novel target of Nek2A in G1/Sarrested cells. J Biol Chem, v. 279, n. 31, p. 32716-27, 2004.

O'Regan L, Fry AM. (2009). The Nek6 and Nek7 protein kinases are required for robust mitotic spindle formation and cytokinesis. Mol Cell Biol. 2009 Jul;29(14):3975-90. Epub 2009 May 4.

O'Connell MJ, Krien MJ, Hunter T. Never say never. The NIMA-related protein kinases in mitotic control. Trends Cell Biol 2003; 13: 221-228 [PMID: 12742165 DOI: 10.1016/.

O'regan L; Blot J; Fry AM. Mitotic regulation by NIMA- related kinases. Cell Div 2007; 2: 25 [PMID: 17727698 DOI:10.1186/1747-1028-2-25] S0962-8924(03)00056-4].

Otto EA; Trapp ML; Schultheiss UT; Helou J; Quarmby LM; Hildebrandt F. (2008). NEK8 mutations affect ciliary and centrosomal localization and may cause nephronophthisis. J Am Soc Nephrol. 2008 Mar;19(3):587-92. Epub 2008 Jan 16.

Paladino P; Marcon E; Greenblatt J; Frappier L. (2014). Identification of herpesvirus proteins that contribute to G1/S arrest. J Virol. 2014 Apr;88(8):4480-92. doi: 10.1128/JVI.00059-14. Epub 2014 Feb 5.

Park S; Lim BB; Perez-Terzic C; Mer G; Terzic A. Interaction of Asymmetric ABCC9-Encoded Nucleotide Binding Domains Determines KATP Channel SUR2A Catalytic Activity. J Proteome Res. 2008, 7, 1721–1728.

Parks WT; Frank DB; Huff C; Renfrew Haft C; Martin J; Meng X; de Caestecker MP; McNally JG; Reddi A; Taylor SI; Roberts AB; Wang T; Lechleider RJ.2001. Sorting nexin 6, a novel SNX, interacts with the transforming growth factor-beta family of receptor serine–threonine kinases. J Biol Chem 276(22): 19332–19339.

Pathak N1; Austin CA; Drummond IA. (2011). Tubulin tyrosine ligase-like genes ttll3 and ttll6 maintain zebrafish cilia structure and motility. J Biol Chem. 2011 Apr 1;286(13):11685-95. doi: 10.1074/jbc.M110.209817. Epub 2011 Jan 24.

Paturle-Lafanechere, L.; Eddé, B.; Denoulet, P.; Van Dorsselaer, A.; Mazarguil, H.; Le Caer, J.P.; Wehland, J. and Job; D. (1991) Characterization of a major brain tubulin variant which cannot be tyrosinated. Biochemistry, 30, 10523-10528.

Paturle-Lafanechere, L.; Manier, M.; Trigault, N.; Pirollet, F.; Mazarguil, H.; and Job, D. (1994). Accumulation of delta 2-tubulin, a major tubulin variant that cannot be tyrosinated, in neuronal tissues and in stable microtubule assemblies. J Cell Sci, 107, 1529-1543.

Pelegrini, A. L.; Moura, D. J.; Brenner, B. L.; Ledur, P. F.; Maques, G. P.; Henriques, J. A.; Saffi, J.; Lenz, G. Nek1 silencing slows down DNA repair and blocks DNA damage-induced cell cycle arrest. Mutagenesis, v. 25, n. 5, p. 447-54, 2010.

Pelka P; Scimè A; Mandalfino C; Joch M; Abdulla P; Whyte P. (2007). Adenovirus E1A proteins direct subcellular redistribution of Nek9, a NimA-related kinase. J Cell Physiol. 2007 Jul;212(1):13-25.

Peris, L.; Thery, M.; Faure, J.; Saoudi, Y.; Lafanechere, L.; Chilton, J.K.; Gordon-Weeks, P.; Galjart, N.; Bornens, M.; Wordeman, L.; et al. (2006). Tubulin tyrosination is a major factor affecting the recruitment of CAP-Gly proteins at microtubule plus ends. J Cell Biol 174, 839-849.

Piao S; Lee SJ; Xu Y; Gwak J; Oh S; Park BJ; Ha NC. (2011). CK1ε targets Cdc25A for ubiquitin-mediated proteolysis under normal conditions and in response to checkpoint activation. Cell Cycle. 2011 Feb 1;10(3):531-7. Epub 2011 Feb 1.

Plessmann, U.; and Weber, K. (1997) Mammalian sperm tubulin: an exceptionally large number of variants based on several posttranslational modifications. J Protein Chem, 16, 385-390.

Polci R; Peng A; Chen PL; Riley DJ; Chen Y. (2004). NIMA-related protein kinase 1 is involved early in the ionizing radiation-induced DNA damage response. Cancer Res. 2004 Dec 15;64(24):8800-3.

Portt L; Norman G; Clapp C; Greenwood M; Greenwood MT (2011) Anti-apoptosis and cell survival: a review. Biochim. Biophys. Acta 1813(1), 238–59.

Posch M; Khoudoli GA; Swift S; King EM; Deluca JG; Swedlow JR. (2010). Sds22 regulates aurora B activity and microtubule-kinetochore interactions at mitosis. J Cell Biol. 4;191(1):61-74.

Pungartnik, C; Kern, MF; Brendel, M and Henriques, JAP.(1999). Mutant allele pso7-1, that sensitizes Saccharomyces cerevisiae to photoactivated psoralen, is allelic with COX11, encoding a protein indispensable for a functional cytochrome c oxidase. Curr. Genet. 36, 124 –129□16. [PMID: 10501934].

Quarmby LM; Mahjoub MR. (2005).Caught Nek-ing: cilia and centrioles. J Cell Sci. 2005 Nov 15;118(Pt 22):5161-9.

Rapley, J.; Baxter, J. E.; Blot, J.; Wattam, S. L.; Casenghi, M.; Meraldi, P.; Nigg, E. A.; Fry, A. M. (2005). Coordinate regulation of the mother centriole component nlp by nek2 and plk1 protein kinases. Mol Cell Biol, v. 25, n. 4, p. 1309-24.

Redeker V; Levilliers N; Schmitter JM; Le Caer JP; Rossier J; Adoutte A; Bre MH (1994) Polyglycylation of tubulin: a posttranslational modification in axonemal microtubules. Science 266: 1688-1691.

Redeker, V.; Le Caer, J.P.; Rossier, J. and Prome, J.C. (1991) Structure of the polyglutamyl side chain posttranslationally added to alpha-tubulin. J Biol Chem, 266, 23461-23466.

Redeker, V.; Melki, R.; Prome, D.; Le Caer, J.P. and Rossier, J. (1992) Structure of tubulin C-terminal domain obtained by subtilisin treatment. The major alpha and beta tubulin isotypes from pig brain are glutamylated. FEBS Lett, 313, 185-192.

Regnard, C.; Audebert, S.; Desbruyeres, Denoulet, P. and Eddé, B. (1998) Tubulin polyglutamylase: partial purification and enzymatic properties. Biochemistry, 37, 8395-8404.

Regnard, C.; Desbruyeres, E.; Denoulet, P. and Eddé, B. (1999) Tubulin polyglutamylase: isozymic variants and regulation during the cell cycle in HeLa cells. J Cell Sci, 112, 4281-4289.

Regnard, C.; Desbruyeres, E.; Huet, J.C.; Beauvallet, C.; Pernollet, J.C. and Eddé, B. (2000) Polyglutamylation of nucleosome assembly proteins. J Biol Chem, 275, 15969-15976.

Regnard, C.; Fesquet, D.; Janke, C.; Boucher, D.; Desbruyeres, E.; Koulakoff, A.; Insina, C.; Travo, P. and Edde, B. (2003) Characterisation of PGs1, a subunit of tubulin polyglutamylase. J Cell Sci, 116, 4181-4190.

Reininger, L.; Tewari, R.; Fennell, C.; Holland, Z.; Goldring, D.; Ranford-Cartwright, L.; Billker, O.; Doerig, C. (2009). An essential role for the Plasmodium Nek-2 Nima-related protein kinase in the sexual development of malaria parasites. J Biol Chem. 2009 Jul 31;284(31):20858-68. doi: 10.1074/jbc.M109.017988. Epub 2009 Jun 2.

Rellos P; Ivins FJ; Baxter JE; Pike A; Nott TJ; Parkinson DM; Das S; Howell S; Fedorov O; Shen QY; Fry AM; Knapp S; Smerdon SJ. (2007) Structure and regulation of the human Nek2 centrosomal kinase. J Biol Chem. 282(9): 6833-6842.

Rhee K; Wolgemuth DJ. (2002) Tcp10 promoter-directed expression of the Nek2 gene in mouse meiotic spermatocytes. Mol Cells. 2002 Feb 28;13(1):85-90.

Riedl, SJ and Shi, Y. (2004). Molecular mechanisms of Caspase regulation during apoptosis. Nat Rev Mol Cell Biol. 5(11): 897-907.

Rocha C; Papon L; Cacheux W; Marques Sousa P; Lascano V; Tort O; Giordano T; Vacher S; Lemmers B; Mariani P; Meseure D; Medema JP; Bièche I; Hahne M; Janke C. (2014). Tubulin glycylases are required for primary cilia, control of cell proliferation and tumor development in colon. EMBO J. 2014 Sep 1. pii: e201488466. [Epub ahead of print].

Rodriguez de la Vega, M.; Sevilla, A.; Hermoso, R.G.; Lorenzo, J.; Tanco, S.; Diez, A.; Fricker, L.D.; Bautista, J.M.and Aviles, F.X. 2007. Nna1-like proteins are active metallocarboxypeptidases of a new and diverse M14 subfamily. Faseb J. 21:851-865.

Rogowski, K., J. van Dijk, M.M. Magiera, C. Bosc, J.-C. Deloulme, A. Bosson, L. Peris, N.D. Gold, B. Lacroix, M.B. Grau, N. Bec, C. Larroque, S. Desagher, M. Holzer, A. Andrieux, M.-J. Moutin, and C. Janke. 2010. A Family of Protein-Deglutamylating Enzymes Associated with Neurodegeneration. Cell. 143:564-578.

Rogowski, K., Juge, F., van Dijk, J., Wloga, D., Strub, J.-M., Levilliers, N., Thomas, D., Bre, M.-H., Van Dorsselaer, A., Gaertig, J. and Janke, C (2009). Evolutionary divergence of enzymatic mechanisms for posttranslational polyglycylation. Cell 137, 1076-1087.

Roig, J.; Mikhailov, A.; Belham, C.; Avruch, J. (2002). Nercc1, a mammalian NIMA-family kinase, binds the Ran GTPase and regulates mitotic progression. Genes Dev. 2002 Jul 1;16(13):1640-58.

Roig, J.; Groen, A.; Caldwell, J.; Avruch, J. (2005). Active Nercc1 protein kinase concentrates at centrosomes early in mitosis and is necessary for proper spindle assembly. Mol Biol Cell, v. 16, n. 10, p. 4827-40.

Rostovtseva, T.K.; Bezrukov, S.M. VDAC regulation: role of cytosolic proteins and mitochondrial lipids. J Bioenerg Biomembr 2008;40:163–170.

Rüdiger, M., Plessman, U., Kloppel, K.D., Wehland, J. and Weber, K. (1992) Class II tubulin, the major brain beta tubulin isotype is polyglutamylated on glutamic acid residue 435. FEBS Lett, 308, 101-105.

Salem, H.; Rachmin, I.; Yissachar, N.; Cohen, S.; Amiel, A.; Haffner, R.; Lavi, L.; Motro, B. (2010). Nek7 kinase targeting leads to early mortality, cytokinesis disturbance and polyploidy. Oncogene. 2010 Jul 15;29(28):4046-57. Epub 2010 May 17.

Schultz, S.J.; Nigg, E.A. (1993) Identification of 21 novel human protein kinases, including 3 members of a family related to the cell cycle regulator nimA of Aspergillus nidulans. Cell Growth Differ. 4(10): 821-830.

Schweigerer, L.; Dockhorn-Dworniczak, B.; Poremba, C. Expression analysis of pediatric solid tumor cell lines using oligonucleotide microarrays. Int J Oncol, v. 20, n. 3, p. 441-51, 2002.

Sdelci, S.; Bertran, M.T.; Roig, J. (2011). Nek9, Nek6, Nek7 and the separation of centrosomes. Cell Cycle. 2011 Nov 15;10(22):3816-7. Epub 2011 Nov 15.

Sdelci, S.; Schütz, M.; Pinyol, R.; Bertran, M.T.; Regué, L.; Caelles, C.; Vernos, I.; Roig, J. (2012). Nek9 phosphorylation of NEDD1/GCP-WD contributes to Plk1 control of γ-tubulin recruitment to the mitotic centrosome. Curr Biol. 2012 Aug 21;22(16):1516-23. doi: 10.1016/j.cub.2012.06.027. Epub 2012 Jul 19.

Shima, F.; Okada, T.; Kido, M.; Sen, H.; Tanaka, Y.; Tamada, M.; Hu, C.D.; Yamawaki-Kataoka, Y.; Kariya, K.; Kataoka, T. (2000). Association of yeast adenylyl cyclase with cyclase-associated protein CAP forms a second Ras-binding site which mediates its Ras-dependent activation. Mol Cell Biol. 2000 Jan;20(1):26-33.

Shimizu, K.; Sawasaki, T. Nek5, a novel substrate for caspase-3, promotes skeletal muscle differentiation by up-regulating caspase activity. FEBS Lett, v. 587, n. 14, p. 2219-25, 2013.

Sklar P; Ripke S; Scott LJ; Andreassen OA; Cichon S; Craddock N; Edenberg HJ; Nurnberger JI Jr; Rietschel M; Blackwood D; Corvin A; Flickinger M; Guan W; Mattingsdal M; McQuillin A; Kwan P; Wienker TF; Daly M; Dudbridge F; Holmans PA; Lin D; Burmeister M; Greenwood TA; Hamshere ML; Muglia P; Smith EN; Zandi PP; Nievergelt CM; McKinney R; Shilling PD; Schork NJ; Bloss CS; Foroud T; Koller DL; Gershon ES; Liu C; Badner JA; Scheftner WA; Lawson WB; Nwulia EA; Hipolito M; Coryell W; Rice J; Byerley W; McMahon FJ; Schulze TG; Berrettini W; Lohoff FW; Potash JB; Mahon PB; McInnis MG; Zöllner S; Zhang P; Craig DW; Szelinger S; Barrett TB; Breuer R; Meier S; Strohmaier J; Witt SH; Tozzi F; Farmer A; McGuffin P; Strauss J; Xu W; Kennedy JL; Vincent JB; Matthews K; Day R; Ferreira MA; O'Dushlaine C; Perlis R; Raychaudhuri S; Ruderfer D; Hyoun PL; Smoller JW; Li J; Absher D; Thompson RC; Meng FG; Schatzberg AF; Bunney WE; Barchas JD; Jones EG; Watson SJ; Myers RM; Akil H; Boehnke M; Chambert K; Moran J; Scolnick E; Djurovic S; Melle I; Morken G; Gill M; Morris D; Quinn E; Mühleisen TW; Degenhardt FA; Mattheisen M; Schumacher J; Maier W; Steffens M; Propping P; Nöthen MM; Anjorin A; Bass N; Gurling H; Kandaswamy R; Lawrence J; McGhee K; McIntosh A; McLean AW; Muir WJ; Pickard BS; Breen G; St Clair D; Caesar S; Gordon-Smith K; Jones L; Fraser C; Green EK; Grozeva D; Jones IR; Kirov G; Moskvina V; Nikolov I; O'Donovan MC; Owen MJ; Collier DA; Elkin A; Williamson R; Young AH; Ferrier IN; Stefansson K; Stefansson H; Thornorgeirsson T; Steinberg S; Gustafsson O; Bergen SE; Nimgaonkar V; Hultman C; Landén M; Lichtenstein P; Sullivan P; Schalling M; Osby U; Backlund L; Frisén L; Langstrom N; Jamain S; Leboyer M; Etain B; Bellivier F; Petursson H; Sigur Sson E; Müller-Mysok B; Lucae S; Schwarz M; Schofield PR; Martin N; Montgomery GW; Lathrop M; Oskarsson H; Bauer M; Wright A; Mitchell PB; Hautzinger M; Reif A; Kelsoe JR; Purcell SM; Psychiatric GWAS Consortium Bipolar Disorder Working Group. (2011). Large-scale genome-wide association analysis of bipolar disorder identifies a new susceptibility locus near ODZ4. Nat Genet. 2011 Sep 18;43(10):977-83. doi: 10.1038/ng.943.

Smith AJ; Lauwaet T; Davids BJ; Gillin FD. (2012) Giardia lamblia Nek1 and Nek2 kinases affect mitosis and excystation. Int J Parasitol. 2012 Apr;42(4):411-9. doi: 10.1016/j.ijpara.2012.03.001. Epub 2012 Mar 13.

Smith, L. A.; Bukanov, N. O.; Husson, H.; Russo, R. J.; Barry, T. C.; Taylor, A. L.; Beier, D. R.; Ibraghimov- Beskrovnaya, O. (2006). Development of polycystic kidney disease in juvenile cystic kidney mice: insights into pathogenesis, ciliary abnormalities, and common features with human disease. J Am Soc Nephrol, v. 17, n. 10, p. 2821-31, 2006.

Sohara E; Luo Y; Zhang J; Manning DK; Beier DR; Zhou J. (2008). Nek8 regulates the expression and localization of polycystin-1 and polycystin-2. J Am Soc Nephrol. 2008 Mar;19(3):469-76. Epub 2008 Jan 30.

Sonn S; Khang I; Kim K; Rhee K. (2004) Suppression of Nek2A in mouse early embryos confirms its requirement for chromosome segregation. J Cell Sci. 2004 Nov 1;117(Pt 23):5557-66. Epub 2004 Oct 12.

Sorensen, C. S.; Melixetian, M.; Klein, D. K.; Helin, K. NEK11: linking CHK1 and CDC25A in DNA damage checkpoint signaling. Cell Cycle, v. 9, n. 3, p. 450-5, 2010.

Spalluto C; Wilson DI; Hearn T. (2012). Nek2 localises to the distal portion of the mother centriole/basal body and is required for timely cilium disassembly at the G2/M transition.. Eur J Cell Biol. 2012 Sep;91(9):675-86. doi: 10.1016/j.ejcb.2012.03.009. Epub 2012 May 19.

Stricker TP; Henriksen KJ; Tonsgard JH; Montag AG; Krausz TN; Pytel P. (2013). Expression profiling of 519 kinase genes in matched malignant peripheral nerve sheath tumor/plexiform neurofibroma samples is discriminatory and identifies mitotic regulators BUB1B, PBK and NEK2 as overexpressed with transformation. Mod Pathol. 2013 Jul;26(7):930-43. doi: 10.1038/modpathol.2012.242. Epub 2013 Feb 1.

Surpili, M. J.; Delben, T. M.; Kobarg, J. Identification of proteins that interact with the central coiled-coil region of the human protein kinase NEK1. Biochemistry, v. 42, n. 51, p. 15369-76, 2003.

Suryavanshi, S.; Eddé, B.; Fox, L.A.; Guerrero, S.; Hard, R.; Hennessey, T.; Kabi, A.; Malison, D.; Pennock, D.; Sale, W.S.; Wloga, D.; Gaertig, J. (2010). Tubulin glutamylation regulates ciliary motility by altering inner dynein arm activity, Curr. Biol. 20: 435–440.

Suzuki T; Okada Y; Semba S; Orba Y; Yamanouchi S; Endo S; Tanaka S; Fujita T; Kuroda S; Nagashima K; Sawa H. (2005). Identification of FEZ1 as a protein that interacts

with JC virus agnoprotein and microtubules: role of agnoprotein-induced dissociation of FEZ1 from microtubules in viral propagation. J Biol Chem 280:24948-56.

Takeno, A.; Takemasa, I.; Doki, Y.; Yamasaki, M.; Miyata, H.; Takiguchi, S.; Fujiwara, Y.; Matsubara, K.; Monden, M. (2008). Integrative approach for differentially overexpressed genes in gastric cancer by combining large scale gene expression profiling and network analysis. Br J Cancer, v. 99, n. 8, p. 1307-15.

Tan, B. C.; Lee, S. C. Nek9, a novel FACT-associated protein, modulates interphase progression. J Biol Chem, v. 279, n. 10, p. 9321-30, 2004.

Tanaka K; Nigg EA. (1999). Cloning and characterization of the murine Nek3 protein kinase, a novel member of the NIMA family of putative cell cycle regulators. J Biol Chem. 1999 May 7;274(19):13491-7.

Thazhath, R.; Liu, C.; and Gaertig, J. (2002). Polyglycylation domain of beta-tubulin maintains axonemal architecture and affects cytokinesis in Tetrahymena. Nature Cell Biol, 4, 256-259.

Thiel C; Kessler K; Giessl A; Dimmler A; Shalev SA; von der Haar S; Zenker M; Zahnleiter D; Stöss H; Beinder E; Abou Jamra R; Ekici AB; Schröder-Kress N; Aigner T; Kirchner T; Reis A; Brandstätter JH; Rauch A. (2011). NEK1 mutations cause short-rib polydactyly syndrome type majewski.Am J Hum Genet. 2011 Jan 7;88(1):106-14.

Treviño MA; García-Mayoral MF; Jiménez MA; Bastolla U; Bruix M.

Tsunoda, N.; Kokuryo, T.; Oda, K.; Senga, T.; Yokoyama, Y.; Nagino, M.; Nimura, Y.; Hamaguchi, M. Nek2 as a novel molecular target for the treatment of breast carcinoma. Cancer Sci, v. 100, n. 1, p. 111-6, 2009.

Upadhya, P.; Birkenmeier, E. H.; Birkenmeier, C. S.; Barker, J. E. (2000). Mutations in a NIMA-related kinase gene, Nek1, cause pleiotropic effects including a progressive polycystic kidney disease in mice. Proc Natl Acad Sci U S A, v. 97, n. 1, p. 217-21.

Uto K; Nakajo N; Sagata N. (1999) Two structural variants of Nek2 kinase, termed Nek2A and Nek2B, are differentially expressed in Xenopus tissues and development. Dev Biol. 1999 Apr 15;208(2):456-64.

van Dijk, J.; Rogowski, K.; Miro, J.; Lacroix, B.; Edde, B. and Janke, C. (2007) A targeted multienzyme mechanism for selective microtubule polyglutamylation. Mol Cell, 26, 437-448.

van Dijk, J.; Miro, J.; Strub, J.M.; Lacroix, B.; van Dorsselaer, A.; Edde, B.; Janke, C. (2008). Polyglutamylation is a post-translational modification with a broad range of substrates, J. Biol. Chem. 283: 3915–3922.

Vaz Meirelles G; Ferreira Lanza DC; da Silva JC; Santana Bernachi J; Paes Leme AF; Kobarg J. (2010). Characterization of hNek6 interactome reveals an important role for its short N-terminal domain and colocalization with proteins at the centrosome. J Proteome Res. 2010 Dec 3;9(12):6298-316. Epub 2010 Oct 22.

Verhey, K.J.; and Gaertig, J. (2007). The Tubulin Code. Cell Cycle, 6, 2152-2160.

Vogel P; Hansen G; Fontenot G; Read R. (2010). Tubulin tyrosine ligase-like 1 deficiency results in chronic rhinosinusitis and abnormal development of spermatid flagella in mice. Vet Pathol. 47(4):703-12. doi: 10.1177/0300985810363485. Epub 2010 May 4.

Wai, D. H.; Schaefer, K. L.; Schramm, A.; Korsching, E.; Van Valen, F.; Ozaki, T.; Boecker, W.; Wang X. The expanding role of mitochondria in apoptosis. Genes Dev 2002;15:2922–2933.

Wang, R.; Song, Y.; Xu, X.; Wu, Q.; Liu, C. The expression of Nek7, FoxM1, and Plk1 in gallbladder cancer and their relationships to clinicopathologic features and survival. Clin Transl Oncol, v. 15, n. 8, p. 626-32, 2013.

Wang, X; Ono, K; Kim, SO; Kravchenko, V; Lin, SC and Han, J (2001) Metaxin is required for tumor necrosis factor-induced cell death. EMBO Rep 2:628–633. [PMID: 11454742].

Wasylyk, C.; Zambrano, A.; Zhao, C.; Brants, J.; Abecassis, J.; Schalken, J.A.; Rogatsch, H.; Schaefer, G.; Pycha, A.; Klocker, H.; Wasylyk, B. (2010). Tubulin tyrosine ligase like 12 links to prostate cancer through tubulin posttranslational modification and chromosome ploidy, Int. J. Cancer. 127: 2542–2553.

Wei R; Ngo B; Wu G; Lee WH. (2011) Phosphorylation of the Ndc80 complex protein, HEC1, by Nek2 kinase modulates chromosome alignment and signaling of the spindle assembly checkpoint. Mol Biol Cell. 2011 Oct;22(19):3584-94. Epub 2011 Aug 10.

Westermann, S.; and Weber, K. (2003). Post-translational modifications regulate microtubule function. Nature Rev Mol Cell Biol, 4, 938-947.

Westwood I; Cheary DM; Baxter JE; Richards MW; van Montfort RL; Fry AM; Bayliss R. (2009) Insights into the conformational variability and regulation of human Nek2 kinase. J Mol Biol. 2009 Feb 20;386(2):476-85. Epub 2008 Dec 24.

White MC; Quarmby LM (2008). The NIMA-family kinase, Nek1 affects the stability of centrosomes and ciliogenesis. BMC Cell Biol, 9:29.

Wloga D; Rogowski K; Sharma N; Van Dijk J; Janke C; Eddé B; Bré MH; Levilliers N; Redeker V; Duan J; Gorovsky MA; Jerka-Dziadosz M; Gaertig J. (2008). Glutamylation on alpha-tubulin is not essential but affects the assembly and functions of a subset of microtubules in Tetrahymena thermophila. Eukaryot Cell. 2008 Aug;7(8):1362-72. doi: 10.1128/EC.00084-08. Epub 2008 Jun 27.5.

Wloga, D.; Webster, D.M.; Rogowski, K.; Bre, M.-H.; Levilliers, N.; Jerka-Dziadosz, M.; Janke, C.; Dougan, S.T.; and Gaertig, J. (2009). TTLL3 Is a tubulin glycine ligase that regulates the assembly of cilia. Dev Cell 16, 867-876.

Wolff, A.; de Nechaud, B.; Chillet, D.; Mazarguil, H.; Desbruyeres, E.; Audebert, S.; Eddé, B.; Gros, F. and Denoulet, P. (1992) Distribution of glutamylated alpha and beta-tubulin in mouse tissues using a specific monoclonal antibody, GT335. Eur J Cell Biol, 59, 425-432.

Wolff, A.; Houdayer, M.; Chillet, D.; de Nechaud, B. and Denoulet, P. (1994) Structure of the polyglutamyl chain of tubulin: occurrence of alpha and gamma linkages between glutamyl units revealed by monoreactive polyclonal antibodies. Biol Cell, 81, 11-16.

Wong RS (2011) Apoptosis in cancer: from pathogenesis to treatment. J. Exp. Clin. Cancer Res. 30, 87.

Wu W; Baxter JE; Wattam SL; Hayward DG; Fardilha M; Knebel A; Ford EM; da Cruz e Silva EF; Fry AM. (2007). Alternative splicing controls nuclear translocation of the cell cycle-regulated Nek2 kinase. J Biol Chem. 2007 Sep 7;282(36):26431-40. Epub 2007 Jul 11.

Wu, Z.; Doondeea, J. B.; Gholami, A. M.; Janning, M. C.; Lemeer, S.; Kramer, K.; Eccles, S. A.; Gollin, S. M.; Grenman, R.; Walch, A.; Feller, S. M.; Kuster, B. (2011).

Quantitative chemical proteomics reveals new potential drug targets in head and neck cancer. Mol Cell Proteomics, v. 10, n. 12, p. M111 011635, 2011.

Wyllie AH; Kerr JF; Currie AR. (1972). Cellular events in the adrenal cortex following ACTH deprivation. J Pathol. 1972 Jan;106(1).

Yang SW; Gao C; Chen L; Song YL; Zhu JL; Qi ST; Jiang ZZ; Wang ZW; Lin F; Huang H; Xing FQ; Sun QY. (2012) Nek9 regulates spindle organization and cell cycle progression during mouse oocyte meiosis and its location in early embryo mitosis. Cell Cycle. 2012 Dec 1;11(23):4366-77. doi: 10.4161/cc.22690. Epub 2012 Nov 16.

Yao J; Fu C; Ding X; Guo Z; Zenreski A; Chen Y; Ahmed K; Liao J; Dou Z; Yao X . (2004). Nek2A kinase regulates the localization of numatrin to centrosome in mitosis. FEBS Lett. 2004 Sep 24;575(1-3):112-8.

Ye B; Li C; Yang Z; Wang Y; Hao J; Wang L; Li Y; Du Y; Hao L; Liu B; Wang S; Xia P; Huang G; Sun L; Tian Y; Fan Z. (2014). Cytosolic carboxypeptidase CCP6 is required for megakaryopoiesis by modulating Mad2 polyglutamylation. J Exp Med. 2014 Oct 20. pii: jem.20141123.

Yim H.; Sung, C. K.; You, J.; Tian, Y.; Benjamin, T. (2011). Nek1 and TAZ interact to maintain normal levels of polycystin 2. J Am Soc Nephrol, v. 22, n. 5, p. 832-7.

Yin, M. J.; Shao, L.; Voehringer, D.; Smeal, T.; Jallal, B. (2003). The serine/ threonine kinase Nek6 is required for cell cycle progression through mitosis. J. Biol. Chem. 278 (52): 52454–52460.

Yissachar, N.; Salem, H.; Tennenbaum, T.; Motro, B. Nek7 kinase is enriched at the centrosome, and is required for proper spindle assembly and mitotic progression. FEBS Lett, v. 580, n. 27, p. 6489-95, 2006.

BAHN, YS; and Sundstrom, P. (2001). CAP1, an Adenylate Cyclase-Associated Protein Gene, Regulates Bud-Hypha Transitions, Filamentous Growth, and Cyclic AMP Levels and Is Required for Virulence of Candida albicans. JOURNAL OF BACTERIOLOGY, p. 3211–3223.

Yu, G.; Swiston, J.; and Young, D. (1994) Comparison of human CAP and CAP2, homologs of the yeast adenylyl cyclase-associated proteins. J. Cell Sci. 107, 1671–1678.

Zempel H; Luedtke J; Kumar Y; Biernat J; Dawson H; Mandelkow E; Mandelkow EM. (2013). Amyloid-β oligomers induce synaptic damage via Tau-dependent microtubule

severing by TTLL6 and spastin. EMBO J. 2013 Nov 13;32(22):2920-37. doi: 10.1038/emboj.2013.207. Epub 2013 Sep 24.

Zhang B; Zhang H; Wang D; Han S; Wang K; Yao A; Li X. (2014). Never in mitosis gene A-related kinase 6 promotes cell proliferation of hepatocellular carcinoma via cyclin B modulation. Oncol Lett. 2014 Sep;8(3):1163-1168. Epub 2014 Jun 30.

Zhou, W.; Yang, Y.; Xia, J.; Wang, H.; Salama, M. E.; Xiong, W.; Xu, H.; Shetty, S.; Chen, T.; Zeng, Z.; Shi, L.; Zangari, M.; Miles, R.; Bearss, D.; Tricot, G.; Zhan, F. (2013). NEK2 induces drug resistance mainly through activation of efflux drug pumps and is associated with poor prognosis in myeloma and other cancers. Cancer Cell, v. 23, n. 1, p. 48-62, 2013.

Ziegelbauer, J.M.; Sullivan, C.S. and Ganem, D. (2009) Tandem array-based expression screens identify host mRNA targets of virus-encoded microRNAs. Nat. Genet. 41, 130–134.

Anexos

Duplo híbrido em leveduras

O sistema duplo híbrido em levedura é uma poderosa metodologia para identificar proteínas que interagem com proteínas alvo *in vivo*, fornecendo a possibilidade do estudo das interações dessas proteínas em sua conformação original. Este sistema também pode ser utilizado para delinear domínios ou resíduos críticos de aminoácidos envolvidos nesta interação (Fields & Sternglanz, 1994; Park *et al.*, 2008). Nesse sentido, é bastante relevante empregar o sistema duplo hibrído em levedura para identificação de possíveis parceiros de interação da Nek5 humana, buscando desvendar as vias de sinalização e os mecanismos funcionais aos quais a Nek5 pode estar envolvida no ciclo celular.

Neste trabalho foi utilizado o sistema Matchmaker GAL4 (Clontech Laboratories, Inc) no qual a isca foi a região C-terminal da proteína Nek5 clonada em vetor PGBKT7 e expressa em fusão com o domínio de ligação ao DNA Gal4 (DNA-BD) e tranformadas em cepa de *S. cerevisiae* Y2HGold *(MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : GAL1UAS–Gal1TATA–His3, GAL2UAS–Gal2TATA–Ade2 URA3 : : MEL1UAS–Mel1TATA AUR1-C MEL1).* A biblioteca Mate & Plate foi comercialmente clonada em vetor pGAD e transformada na cepa de *S. cerevisiae* Y187 *(MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4*Δ, *met–, gal80*Δ, *URA3 : : GAL1*UAS-*GAL1*TATA-*lacZ;* Harper *et al*., 1993; AD = Fields & Song, 1989; Chien *et al.*, 1991).

Neste sistema, quando a isca interage com alguma proteína da biblioteca o DNA-BD e AD ficam próximos o suficiente para ativar fatores de transcrição de quatro genes repórter independentes (*AUR1-C, ADE2, HIS3*, e *MEL1*) aumentando a seletividade do sistema. A AUR1-C é uma versão dominante do gene AUR1 que codifica a enzima *inositol phosphoryl ceramide synthase* e é expressa em Y2HGold em resposta a interações proteína-proteína, em *Saccharomyces cerevisiae* e sua expressão confere resistência (AbAr) a droga Aureobasidina A. Adicionalmente, Y2HGold é incapaz de crescer em meio ausente em histidina, entretanto, quando isca e presa interagem é ativado o gene His3 permitindo que a célula biosintetize este aminoácido, assim como ocorre para o gene ADE2 que codifica para adenina. MEL1 é outro gene repórter deste sistema que codifica a enzima α-galactosidase. Em resposta interação entre isca e presa, MEL1 é expressa e a colônia se torna azul em presença do substrato cromogênico X-α-Gal. A biblioteca Mate & Plate (Clontech

Laboratories, Inc) é normalizada, e dessa forma, uma menor quantidade de clones é necessário para ser detectado, uma vez que a representação de cada gene é equalizada, diminuindo significativamente a abundância de genes *housekeeping*. Este sistema utiliza a habilidade natural de linhagens de leveduras haplóides como Y187 e Y2HGold para interagir uma com a outra para formar uma célula diplóide, favorecendo a introdução de toda a biblioteca.

Para a conjugação das cepas Y187 contendo a biblioteca e Y2HGold contendo Nek5 clonado em pGBKT7 foi coletada uma colônia de Y2H+pGBKT7-Nek5 e inoculada em 50mL de SD-W em meio líquido e incubada a 30º C a 250 rpm até atingir OD600 de 0,8. As células foram centrifugadas a 1000 g por 5 min e o pellet ressupendido em 10mL de SD-W. Em seguida foi adicionado a estas células 1mL de Biblioteca Universal Mate & Plate (Clontech) e incubadas a 30°C por 30 horas com agitação de 40 rpm. Após este período as células foram centrifugadas, ressupendidas 10mL de 0,5xYPDA/Kan líquido, plaqueadas em placas SD-WL contendo aureobasidina, antibiótico utilizado para o seleção de clones que tiveram interação proteína-proteína, e incubadas em estufa a 30°C até o surgimento de colônias. As colônias que cresceram em placas SD-WL Aureobasidina foram repicadas para placas mais seletivas SD –WLAH contendo aureobasidina e X-α-Gal (Fig. 5). Para identificar o cDNA clonado no vetor presa (biblioteca) foi realizado o isolamento de DNA plasmidial de levedura sugerido pelo Clontech dos clones positivos. O DNA plasmidial foi amplificado em *E. coli* (DH5α), utilizando-se o antibiótico de seleção do plasmídeo da biblioteca, e então seqüenciado com os *primers* (sense) indicados pelo fabricante da biblioteca. As seqüências obtidas foram submetidas à análise no banco de dados GenBank (NCBI, http://www.ncbi.nlm.nih.gov/) e analisadas através do programa BLASTX (Altschul *et al*., 1997).

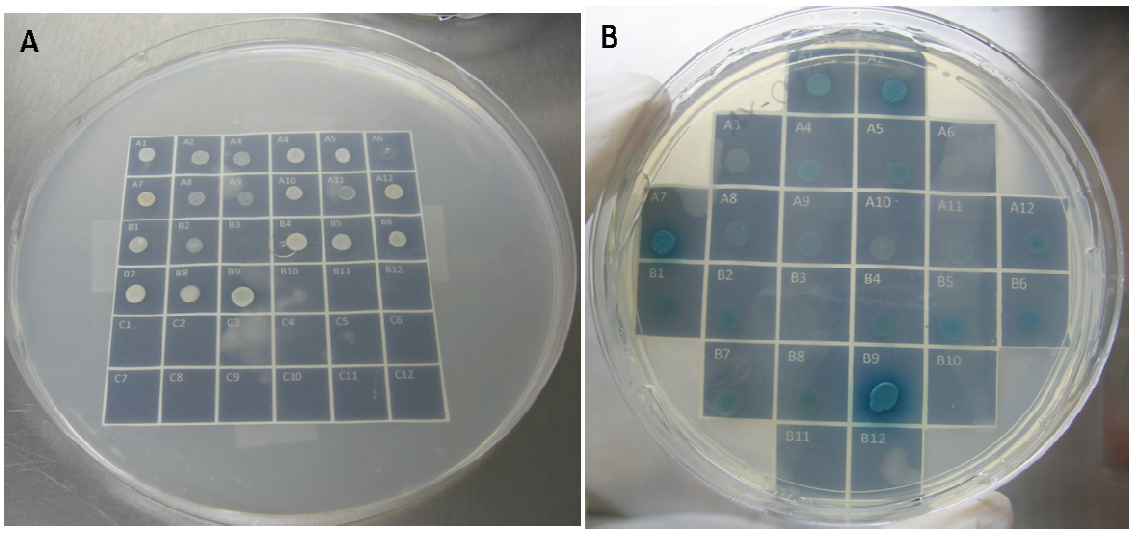


Figura 5: Colônias obtidas pelo sistema de duplo híbrido utilizando como isca a proteína hNEk5. A) Colônias que cresceram em meio seletivo SD-WL contendo aureobasidina. O crescimento em aureobasidina representa que as colônias ativaram o gene repórter AuriC. B) Colônias que cresceram em meio ainda mais seletivo SD-WLAH contendo aureobasidina e X-α-Gal.. Além do gene repórter AuriC foram ativados os genes Mel1, His3 e Ade2 como indicadores de interação entre as proteínas. O clone B9 é o controle positivo contendo a proteína p53 e T7.

A tabela 3 apresenta as proteínas identificadas por duplo híbrido como interactoras de Nek5. Ao total 10 proteínas foram identificadas e estão envolvidas em diferentes processos celulares. Três destas proteínas estão localizadas ou envolvidas com processos mitocondriais e foram abordadas no artigo II do presente trabalho. São elas Metaxina-2 (MTX2), proteína de montagem do citocromo c oxidase (COX11) e fator 1 de transcrição associado a Bcl2 (BCLAF1).

A proteína Metaxina-2 está ligada a membrana mitocondrial externa e juntamente com a Metaxin-1 sugere-se que estejam envolvidas na importação de proteínas para a mitocôndria (Armstrong & Bornstein, 1999). Ela tem seus níveis de expressão aumentados em diferentes tipos de câncer como o de próstata (Lexander et. al, 2006) e é requerida para a morte celular induzida por TNF (Wang et al., 2001). A MTX2 esta ainda envolvida na biogênese do canal seletivo de voltagem dependente de ânion (VDAC) que é responsável pela troca de metabólitos entre o citosol e mitocondria e tem sido correlacionado a diferentes formas de apoptose (Kozjak-Pavlovic et al., 2007 e Cheng et al., 2003). A proteína Cox 11, presente na membrana mitocondrial interna, é essencial para o

crescimento respiratório e está envolvida na biogênese do centro Cu_B da enzima citocromo c oxidase envolvida no passo final da cadeia respiratória mitocondrial, no qual o oxigênio é reduzido a água (Greiner et al., 2008). Ela é responsável ainda pela entrega de cobre (Cu) ao centro Cu_B do citocromo c oxidase, (Carr et al., 2002). A proteína BCLAF1, por sua vez, interage com membros antiapoptóticos da família BCL2 atuando como indutor de apoptose (Kasof et al., 1999). Ela está reduzida ou ausente em células tumorais, sendo considerada agente supressor de tumor (Sarras et al., 2010). BCLAF1 também participa como repressor de transcrição e em processos como desenvolvimento pulmonar (McPearson et al., 2009), ativação de células T e controle da infecção lítica do sarcoma de Kaposi (Ziegelbauer et al., 2009).

Tabela 3: Proteínas que interagem com hNek5 identificadas por Duplo Híbrido.

Proteína (Blastx)	Gene	Número de acesso	Resíduos identificados	Frame	Cobertura %	Processo biológico
metaxin 2	MTX2	NP_006545.1	4-181, 207-261	1	58	Processos celulares metabólicos, direcionamento de proteínas para a mitocôndria
sorting nexin 6	SNX6	EAW65916.1	9-289	1	74	Comunicação celular, transporte de proteínas intracelular
UPF0731 protein C6orf225	C6orf225	NP_001028736.1	1-80.	1	22	
DDB1- and CUL4-associated factor 10	DCAF10	XP_002819646.1	97-221	2	37	Ubiquitinação de proteínas
CAP, adenylate cyclase-associated protein, 2 (yeast)	CAP2	CAI12250.1	131-410	1	87	Ativação de adenilato ciclase, estabelecimento ou manutenção da polaridade celular, transdução de sinal
BCL2-associated transcription factor 1	BCLAF1	NP_001070909.1	677-725	1	13	Indução de apoptose, regulador negative da transcrição, regulador positive da via intrínseca da apoptose, regulador de resposta a danos de DNA
SET domain, bifurcated 1	SETDB1	AAH28671.1	602-670	3	35	Regulação da transcrição, transcrição DNA dependente
tubulin polyglutamylase TTLL4	TTLL4	NP_055455.3	895-1189	3	81	Poliglutamilação de proteínas
DNA	TOP2	AAC774	1283-1347	1	56	Mudança topológica de DNA,

topoisomerase II beta	B	32.1				transição G2/M do ciclo celular, segregação de cromátides irmãs, *checkpoint* de ciclo celular
cytochrome c oxidase assembly protein COX11,	COX 11	XP_0028 27408.1	91-287	2	51	Montage do complex IV da cadeia respiratória, troca de gases respiratórios

Outra importante proteína identificada foi a tubulina poliglutamilase (TTLL4) que está envolvida na glutamilação de proteínas. A caracterização desta proteína pode ser observada no artigo III desta tese. A poliglumatamilação é uma nova modificação pós traducional no qual cadeias de glutamato são formadas na proteína (Eddé et al., 1990; Redeker et al., 1994). Domínios ricos em repetições de glutamina podem formar estruturas em α-hélices e *coiled-coil* levando a agregação de proteínas. Esta agregação está associada a diversas doenças como formação do peptídeo β-amilóide e mal de Alzheimer (Michelitsch & Weissman, 2000, Link & Saldi, 2012). Repetições de glutamina podem ainda ter um "papel natural" na indução de morte celular que se torna desregulada em doenças neurodegenerativas. Além das tubulinas, as proteínas TTLL4 e TTLL5 são capazes de glutamilar diversas proteínas como PELP1 cuja glutamilação esta envolvida com o processo de remodelamento da cromatina em células de câncer pancreático (Kashiwaya et al., 2010). Além das funções anteriormente descritas. TTLL4 ainda participa do processo de poliglutamilação de tubulinas, no qual foi primeiramente descoberto este tipo de modificação. A poliglutamilação ocorre na região C-terminal, responsável pela ligação de diversas proteínas motoras e associadas aos microtúbulos. Assim, acredita-se que a poliglutamilação de tubulinas pode modular a associação dos microtúbulos com estas proteínas motoras (Bonnet *et al.*, 2001; Boucher *et al.*, 1994; Larcher *et al.*, 1996).

Foram identificadas ainda as proteínas Sortin Nexin 6 (SNX6), Fator 10 associado a DDB1- e CUL4 (DCAF10), Proteína 2 associada a adenilato ciclase (CAP2) e DNA topoisomerase II beta (TOPO2B). A SNX6 pertence a família das proteínas SNX envolvidas com o transporte de vesículas e *sorting* de proteínas (Worby and Dixon, 2002). Okada e colaboradores (2010) identificaram a interação de SNX6 com a BACE1 que promove a primeira clivagem do precursor β-amilóide para formação do peptídeo β-amilóide na doença de Alzheimer. É possível observar que tanto TTLL4 quanto SNX6

parecem estar relacionadas ao surgimento de doenças como a de Alzheimer. Neste contexto no futuro pretendemos avaliar se hNek5 exerce algum tipo de função neste tipo de patologia. A SNX6 também está envolvida no transporte intracelular de receptores de membrana e interage com o TGFβ, EGF e insulina. Está localizada no citoplasma em um complexo protéico denominado retrômero que medeia o transporte retrógrado de membrana cargo dos endossomos para o trans-Golgi (Parks et al., 2001). A proteína CAP2, também identificada por duplo híbrido, possui pouca informação em bancos de dados, mas, acredita-se que ela tenha funções similares a CAP1, outro membro da família com a qual compartilha 64% de identidade (Yu et al., 1994). CAP1 está superexpressa em câncer pancreático e está envolvida na motilidade celular de cânceres (Yamazaki et al., 2009). As CAPs são moléculas multifuncionais e em *Candida albicans* CAP1 regula a transição *Bud-Hypha*, o crescimento de filamentos e os níveis de cAMP necessários para sua virulência. (Bahn & Sundstrom, 2001). Em eucariotos elas estão relacionadas ao remodelamento do citoesqueleto de actina (Deeks et al., 2007) e ativação dependente de Ras regulando crescimento celular e diferenciação (Shima et al., 2000).

Para entender melhor as proteínas identificadas em um contexto mais global submetemos os cromatogramas obtidos do sequênciamento das presas à plataforma *Integrated Interactome System* (http://bioinfo03.ibi.unicamp.br/lnbio/interactome/) desenvolvida pela Dra. Gabriela Meirelles (Carazzolle et al., 2014). Esta plataforma utiliza a ferramenta de alinhamento Blast (banco de dados do NCBI) para a análise das sequências. Como pode ser observado na figura 6 o programa compara diferentes bancos de dados de interações, criando uma rede em nível primário da proteína alvo (hNek5 em vermelho) com as proteínas identificadas pelo duplo híbrido e interações descritas nos bancos de dados (círculos em azul). Além disso, é possível ainda, formar uma rede em segundo nível no qual proteínas que interagem com as iscas identificadas também são adicionadas à rede (círculos em verde). Como as informações sobre hNek5 e seus parceiros são restritas, a utilização da plataforma não permitiu adicionar informações quanto a parceiros diretos da hNek5, mas nos permitiu agrupar estes parceiros, assim como descobrir proteínas que interagem com mais de uma proteína como foi o caso de SRRM1, HDAC1, HDAC2 e SUMO3, sugerindo vias convergentes de ação das proteínas identificadas.

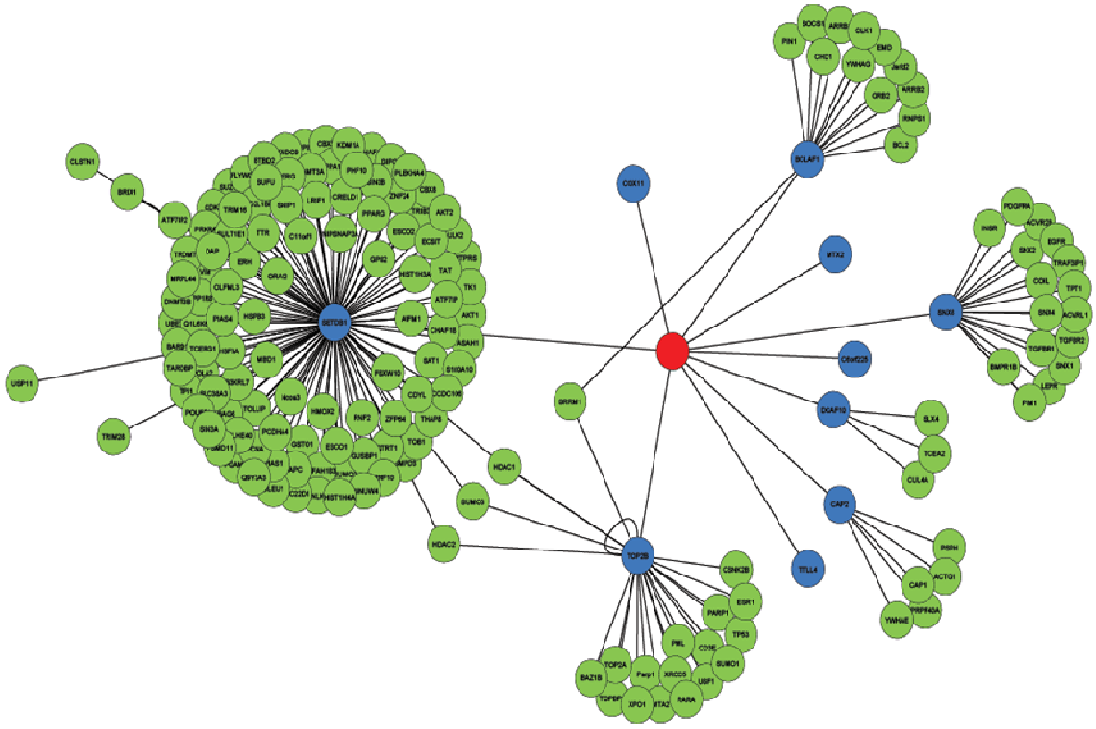


Figura 6: Rede de interações da proteína humana Nek5. A rede de interações de hNek5 possui 10 proteínas que interagiram diretamente com hNek5 por duplo híbrido (nodos azuis). Os 164 nodos verdes representam proteínas que interagem com estes parceiros de acordo com o programa *Integrated Interactome System* desenvolvido por nosso grupo.

Considerando os resultados obtidos pelo sistema de duplo híbrido foi utilizada a microscopia confocal para observar se as proteínas identificadas colocalizavam com hNek5, tornando possível a interação entre estas. A colocalização destas proteínas pode ser observada nas figuras 7 e 8 e foram realizadas em células de osteosarcoma, U2OS (Fig. 7), e de câncer de mama, MCF7 (Fig. 8). hNek5 endógena foi marcada em vermelho com Alexa-594, ao passo que as presas foram marcadas em verde com Alexa-488. A imagem sobreposta (*merge*) mostra uma ampla distribuição da hNek5 no núcleo e citoplasma. A análise de colocalização foi realizada pelo programa *Zen Light blue edition* e representa as regiões da célula onde isca e presa colocalizaram. A partir destas imagens é possível afirmar que hNek5 colocaliza com todas as proteínas testadas em diferentes regiões da célula. A proteína Topoisomerase IIB, apesar de ser nuclear apresentou forte colocalização com hNek5. DCAF e MTX2 são proteínas citoplasmáticas e mitocondriais respectivamente,

e também apresentaram significativa colocalização com hNek5. Desta forma é possível observar que hNek5 apresenta uma ampla distribuição nas células, sendo encontrada no núcleo, citoplasma e mitocôndrias até o presente momento.

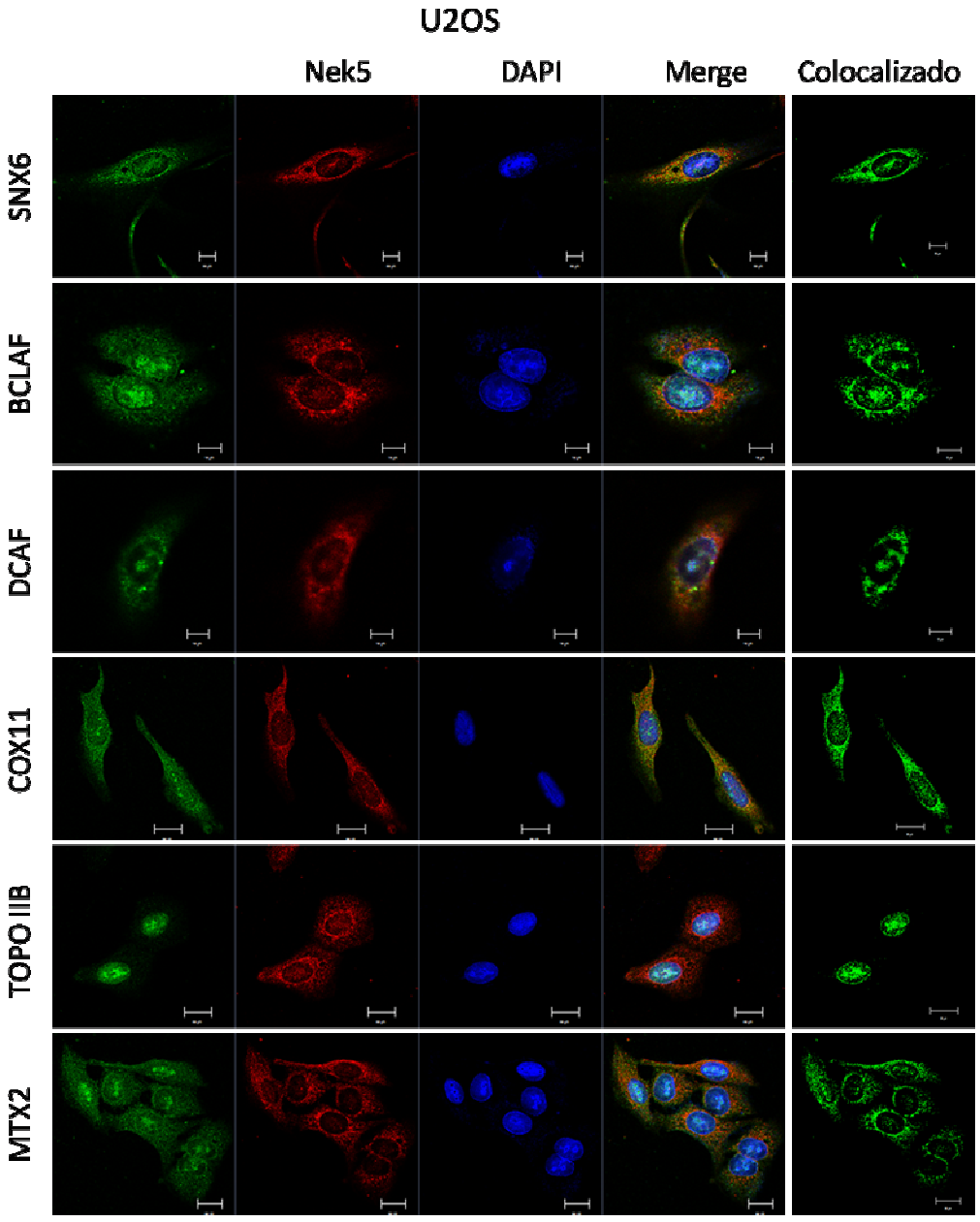


Figura 7: hNek5 colocalizou com todas as proteínas testadas na linhagem celular humana de osteosarcoma, U2OS. Em vermelho hNek5 marcada com Alexa 594 e em verde, presas identificadas ao lado esquerdo da figura, marcadas com Alexa 488. Os núcleos foram corados com Hoenchst. A coluna colocalização representa a imagem gerada pelo programa Zen 2009 da colocalização de hNek5 e suas respectivas presas.

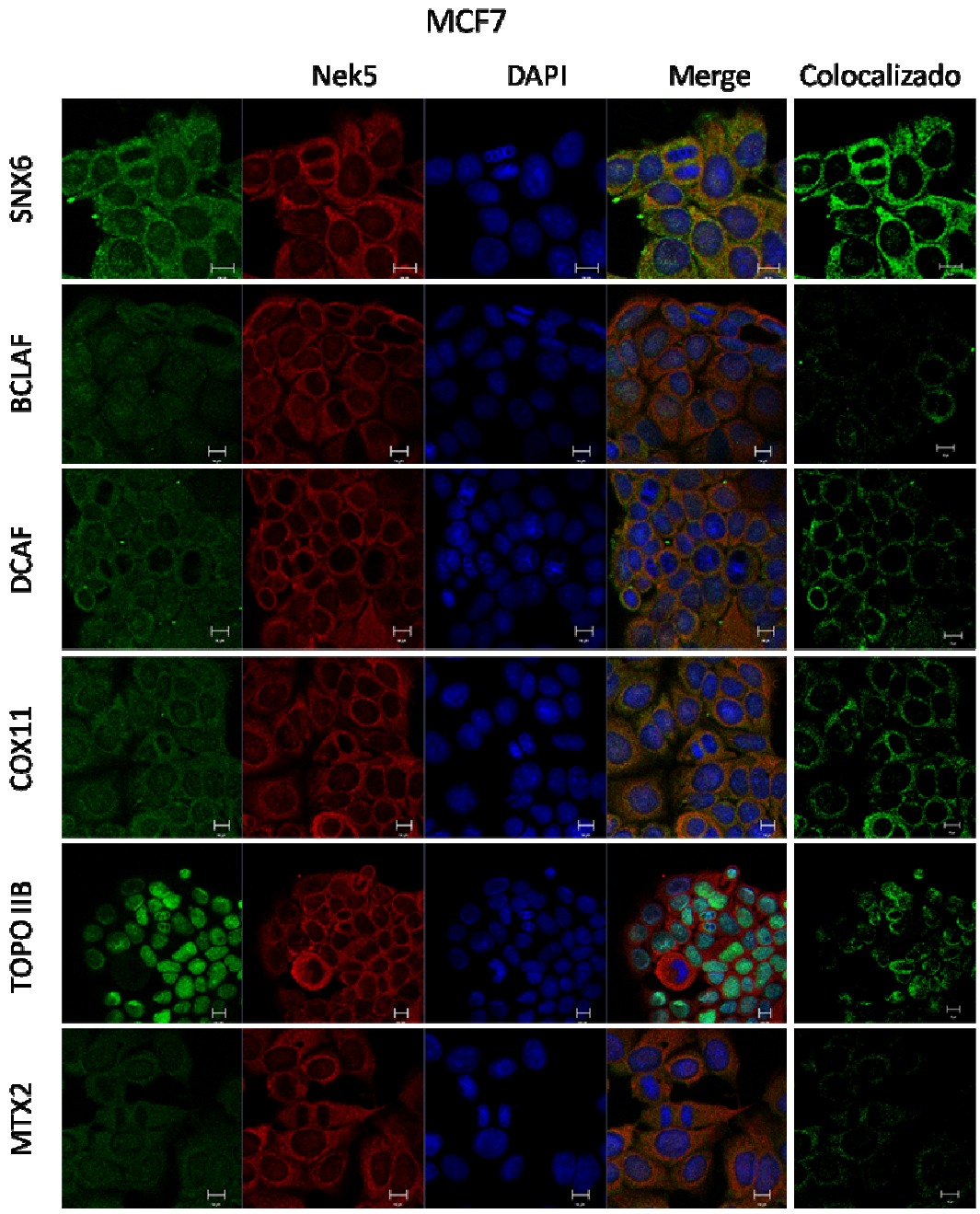


Figura 8: hNek5 colocalizou com todas as proteínas testadas na linhagem celular humana de adenocarcinoma de mama, MCF7. Em vermelho hNek5 marcada com Alexa 594 e em verde, presas identificadas ao lado esquerdo da figura, marcadas com Alexa 488. Os núcleos foram corados com Hoenchst. A coluna colocalização representa a imagem gerada pelo programa Zen 2009 da colocalização de hNek5 e suas respectivas presas.

Fracionamento e Espectrometria de Massas

O estudo de moléculas que interagem com uma proteína pode ser fundamental para a compreensão da sua função. Através da proteômica, muitos avanços têm sido feitos em diferentes áreas. As ferramentas utilizadas para a análise do proteoma geralmente incluem inicialmente a extração do material proteico de um modelo biológico que pode ter sofrido uma condição diferente da normal, como por exemplo, a super expressão de proteínas alvo. As proteínas de interesse são separadas do total para identificação através de técnicas mais refinadas como espectrometria de massas.

O ensaio de duplo híbrido permitiu a identificação de algumas proteínas parceiras de hNek5. Muito foi avançado a partir destes resultados, como por exemplo, a descoberta da localização mitocondrial de hNek5 e seu envolvimento em processos como morte celular e poliglutamilação. Tendo em vista a presença de hNek5 no microambiente mitocondrial utilizamos da estratégia de imunoprecipitação para verificar a interação *in vitro* de extratos protéicos provenientes de células expressando estavelmente hNek5-Flag e vetor Flag vazio. As células em questão foram fracionadas utilizando o kit Qproteome® *Mitochondria Isolation* (Qiagen) e as proteínas das frações de citoplasma e mitocôndria foram submetidos a imunoprecipitação com o resina anti-FLAG M2® (Sigma). As proteínas imunoprecipitadas e os imunocomplexos gerados foram eluídos da matriz sólida com tampão de amostra contendo SDS, aquecidas a 100°C, corridas em gel SDS-PAGE, coradas com prata e coomassie e analisadas por espectrometria de massas. Este experimento foi realizado em triplicata e a figura 9 representa as amostras de citoplasma e mitocôndria de ambas as linhagens celulares que foram separadas por SDS-PAGE e coradas com prata (Fig. 9). Os imunoprecipitados foram digeridos com tripsina e avaliados no espectrômetro de massa do tipo *Eletrospray Q-Tof* no modo positivo para a obtenção de espectros do tipo MS-MS. A análise dos dados foi feita utilizando o software MASCOT e o Swiss prot como banco de dados, disponível na internet, considerando apenas sequências humanas. Amostras do clone estável apenas para o vetor FLAG vazio foram utilizadas como controle e apenas as proteínas que não apareceram em nenhuma das triplicatas do grupo FLAG e que apareceram pelo menos em uma das triplicatas de hNek5 FLAG foram utilizadas nas análises subseqüentes. As proteínas que interagem direta ou indiretamente com hNek5 nas

frações de citoplasma estão demonstradas na tabela 4 e as proteínas das frações mitocondriais apresentadas na tabela 5.

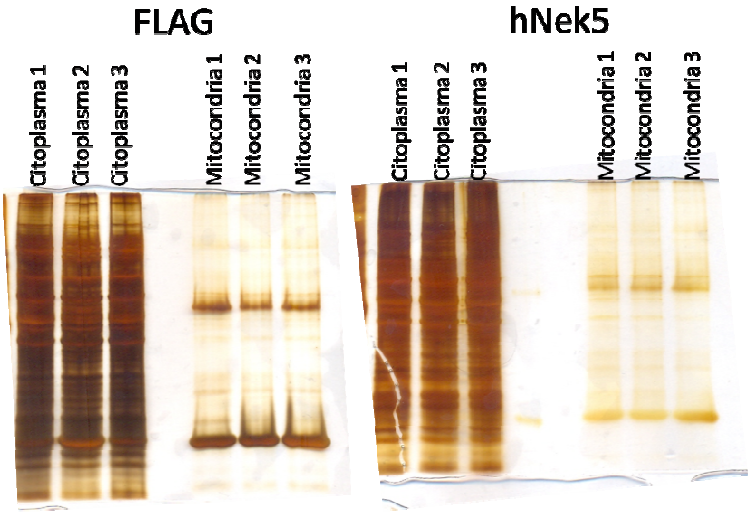


Figura 9: Gel de eletroforese em poliacrilamida SDS-PAGE das amostras de imunoprecipitação submetidas a espectrometria de massas. As amostras foram aplicadas em triplicata, fracionadas, submetidas a digestão com tripsina e analisadas por espectrometria de massas.

Tabela 4: Proteínas identificadas na fração mitocondrial de células hNek5-FLAG. - Identificação por espectrômetro de massas das proteínas detectadas na fração mitocondrial de células hNek5-FLAG após imunoprecipitação.

Acesso	Descrição	Amostra 1	Amostra 2	Amostra 3	MW [kDa]	calc. pI
P04406	Glyceraldehyde-3-phosphate dehydrogenase	2	1	3	36,0	8,46
P29692	Elongation factor 1-delta	7	2	1	31,1	5,01
P54819-5	Isoform 5 of Adenylate kinase 2	3	2	3	24,6	6,70
P60174-4	Isoform 4 of Triosephosphate isomerase	2		1	17,9	5,58
P30041	Peroxiredoxin-6	5	3	1	25,0	6,38
P21912	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit	3	4	4	31,6	8,76
M0R208	ATP-dependent Clp protease proteolytic subunit	3	1	2	20,6	5,50
P52597	Heterogeneous nuclear ribonucleoprotein F	3	1		45,6	5,58
F8W020	Nucleosome assembly protein 1-like 1	3			24,4	4,51
M0R0Z3	General transcription factor IIF subunit 1	2			14,7	9,88
O60506-4	Isoform 4 of Heterogeneous nuclear ribonucleoprotein Q	5	3	3	58,7	7,56
Q00688	Peptidyl-prolyl cis-trans isomerase FKBP3	3	1		25,2	9,28
P43686-2	Isoform 2 of 26S protease regulatory subunit 6B	4			43,5	5,26
F5H3X9	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	7	1	1	59,2	5,68
E9PM69	26S protease regulatory subunit 6A	5			44,3	5,05
P06748-2	Isoform 2 of Nucleophosmin	4	1	1	29,4	4,61
Q5RKV6	Exosome complex component MTR3	2			28,2	6,28
E9PDU6	Calponin-3	2			20,2	9,42
K7EL96	Perilipin-3	2			18,1	5,34
P12004	Proliferating cell nuclear antigen	5			28,8	4,69
Q9NTK5	Obg-like ATPase 1	5			44,7	7,81
K7ENH2	Proteasome activator complex subunit 3	2			20,6	5,34
E9PJ81	UBX domain-containing protein 1	2			32,6	5,14

Acesso	Descrição	Amostra 1	Amostra 2	Amostra 3	MW [kDa]	calc. PI
Q5VU61	Tropomyosin alpha-3 chain	3			26,3	4,84
K7EMD6	Small glutamine-rich tetratricopeptide repeat-containing protein alpha	2			17,8	4,75
H3BU31	60S ribosomal protein L4	3	1		19,0	11,72
F5GZ27	Lon protease homolog	6	4	4	85,6	6,04
Q12904	Aminoacyl tRNA synthase complex-interacting multifunctional protein	4			34,3	8,43
Q5SQT6	Inorganic pyrophosphatase	3			20,0	5,25
P50454	Serpin H1	3	4		46,4	8,69
O43399-6	Isoform 6 of Tumor protein D54	2			17,5	6,60
Q12906-5	Isoform 5 of Interleukin enhancer-binding factor 3	4	3	2	74,6	8,24
P49006	MARCKS-related protein	2	4	2	19,5	4,67
Q99614	Tetratricopeptide repeat protein 1	3			33,5	4,84
P50897	Palmitoyl-protein thioesterase 1	3	2		34,2	6,52
B5MCY6	Protein phosphatase 1 regulatory subunit 7	2			25,6	5,02
J3QL05	Serine/arginine-rich-splicing factor 2	2			15,1	10,96
Q9Y3Y2-4	Isoform 3 of Chromatin target of PRMT1	2			21,9	11,97
P31948	Stress-induced-phosphoprotein 1	4	2		62,6	6,80
Q9UBV8	Peflin	3			30,4	6,54
Q92820	Gamma-glutamyl hydrolase	2	1		35,9	7,11
P31942-3	Isoform 3 of Heterogeneous nuclear ribonucleoprotein H3	2	1	1	31,5	7,31
K7EPL2	SUMO-activating enzyme subunit 2	2			29,8	6,42
Q9UHD9	Ubiquilin-2	4			65,7	5,22
Q14257	Reticulocalbin-2	2	1	1	36,9	4,40
P36543	V-type proton ATPase subunit E	2			26,1	8,00
Q9UNZ2-4	Isoform 2 of NSFL1 cofactor p47	2			37,3	5,11
Q92522	Histone H1x	2			22,5	10,76
P20645	Cation-dependent mannose-6-phosphate receptor	2			31,0	5,83

Acesso	Descrição	Amostra 1	Amostra 2	Amostra 3	MW [kDa]	calc. pI
H0YI98	Dynactin subunit 2	2			29,3	6,80
H0Y2Y8	Zyxin	3			57,6	6,87
K7EL20	Eukaryotic translation initiation factor 3 subunit G	2			29,4	5,41
Q9HC38-3	Isoform 3 of Glyoxalase domain-containing protein 4	2			21,2	5,66
O14654	Insulin receptor substrate 4	6	4		133,7	8,44
P00492	Hypoxanthine-guanine phosphoribosyltransferase	2			24,6	6,68
F8WD96	Cathepsin D light chain	2	2	1	30,0	7,42
Q07866-3	Isoform G of Kinesin light chain 1	4			64,3	6,30
Q06323	Proteasome activator complex subunit 1	2			28,7	6,02
Q96AE4	Far upstream element-binding protein 1	4	1		67,5	7,61
Q96AG4	Leucine-rich repeat-containing protein 59	2	1	1	34,9	9,57
Q14677-3	Isoform 3 of Clathrin interactor 1	3			70,2	6,58
Q08J23-2	Isoform 2 of tRNA (cytosine(34)-C(5))-methyltransferase	3		1	82,3	7,01
C9JH18	PERQ amino acid-rich with GYF domain-containing protein 2	2			16,8	9,79
Q9UMX0-2	Isoform 2 of Ubiquilin-1	4			59,2	5,10
Q8NBS9-2	Isoform 2 of Thioredoxin domain-containing protein 5	2	2		36,2	5,47
F8W6E4	Glycerol-3-phosphate dehydrogenase	2		2	55,7	6,25
Q8N1G4	Leucine-rich repeat-containing protein 47	2	1	1	63,4	8,28
Q8N8S7-2	Isoform 2 of Protein enabled homolog	2			63,9	6,43
B7Z6M1	Plastin-3	4			65,6	6,30
P18206-2	Isoform 1 of Vinculin	2		3	116,6	6,09
Q99689	Fasciculation and elongation protein zeta-1	3			45,1	4,30
P78347-2	Isoform 2 of General transcription factor II-I	3	2		107,9	7,94
M0QYL4	A-kinase anchor protein 8	2			39,4	5,06
Q5T757	Serine/arginine-rich-splicing factor 11	2			48,5	10,54
Q8WZA9	Immunity-related GTPase family Q protein	2			62,7	4,88
B4DXI8	26S proteasome non-ATPase regulatory subunit 7	2			28,3	7,62

Acesso	Descrição	Amostra 1	Amostra 2	Amostra 3	MW [kDa]	calc. pI
Q4VXW4	Opioid growth factor receptor	2			52,4	5,20
B4DME9	Kinesin light chain 4	2			59,6	6,15
Q9Y376	Calcium-binding protein 39	2			39,8	6,89
Q99543-2	Isoform 2 of DnaJ homolog subfamily C member 2	2			65,9	9,07
Q5TFE4	5'-nucleotidase domain-containing protein 1	2			51,8	6,35
C9JZG1	Eukaryotic translation initiation factor 3 subunit B	3			33,4	4,36
O15355	Protein phosphatase 1G	3			59,2	4,36
Q7KZF4	Staphylococcal nuclease domain-containing protein 1	2		1	101,9	7,17
Q7Z417	Nuclear fragile X mental retardation-interacting protein 2	2			76,1	8,70
Q16822	Phosphoenolpyruvate carboxykinase [GTP]	2			70,7	7,62
Q15007	Pre-mRNA-splicing regulator WTAP	2			44,2	5,19
P46821	Microtubule-associated protein 1B	2	1	1	270,5	4,81
P48634	Protein PRRC2A	2	2		228,7	9,45
Q96QC0	Serine/threonine-protein phosphatase 1 regulatory subunit 10	2			99,0	9,17
P12270	Nucleoprotein TPR	2	1		267,1	5,02
O75369-5	Isoform 5 of Filamin-B	2	1		230,1	5,54

Tabela 5: Proteínas identificadas na fração citoplasmática de células hNek5-FLAG. - Identificação por espectrômetro de massas das proteínas detectadas na fração citoplasmática de células hNek5-FLAG após imunoprecipitação.

Acesso	Descrição	ΣCoverage	Amostra 1	Amostra 2	Amostra 3	MW [kDa]
E5RHC7	Eukaryotic translation initiation factor 3 subunit H	41,30	2	1	1	4,3
B7Z6P0	Cytosolic Fe-S cluster assembly factor NUBP2	40,77		1	1	13,8
M0R210	40S ribosomal protein S16	39,53	4	3	2	14,4
Q5T7C4	High mobility group protein B1	37,97	1	4	1	18,3
Q96SI1	BTB/POZ domain-containing protein KCTD15	36,40	1	5	3	31,9
P62829	60S ribosomal protein L23	32,14	4	3		14,9
Q5T0R7	Adenylyl cyclase-associated protein 1	29,89			4	19,2
P62805	Histone H4	29,13	2	3		11,4
A8K8G0	Hepatoma-derived growth factor	25,96	1	1	2	22,9
E3W974	Aspartyl aminopeptidase	25,53	2			5,1
P62280	40S ribosomal protein S11	24,68	2	3		18,4
F5H018	GTP-binding nuclear protein Ran	24,12	2	5	3	22,5
P62081	40S ribosomal protein S7	23,20	5	7	4	22,1
H0Y9G8	Septin-11	23,17		1	1	9,8
E9PBS1	Phosphoribosylaminoimidazole carboxylase	23,00	7	5	1	45,6
Q9Y5Z4	Heme-binding protein 2	22,93	2	1	3	22,9
E7EX53	Ribosomal protein L15	22,56		2	1	15,7
C9JLN6	Asparagine synthetase [glutamine-hydrolyzing]	22,22	1	1	2	7,5
F5H218	Cell division cycle and apoptosis regulator protein 1	21,25		1		8,9
J3KPD3	RNA binding motif protein 7, isoform CRA_c	20,97		2	3	30,6
H3BQA6	Cyclin-D1-binding protein 1	20,97		1		7,1
Q9Y580	RNA-binding protein 7	20,68		2	3	30,5

Acesso	Descrição	ΣCoverage	Amostra 1	Amostra 2	Amostra 3	MW [kDa]
P78371	T-complex protein 1 subunit beta	20,19	6	4	3	57,5
Q8WXX5	DnaJ homolog subfamily C member 9	20,00	1	1	3	29,9
P30050	60S ribosomal protein L12	19,39		1	1	17,8
E9PP76	Superoxide dismutase [Cu-Zn]	19,12		1	1	7,3
Q5QPB1	AN1-type zinc finger protein 5	18,96	1		2	22,5
F8VQS0	Ataxin-2	18,30	1	1	1	17,5
Q9H446	RWD domain-containing protein 1	18,11	2	1	2	27,9
B4DP11	Prostaglandin E synthase 3	17,99		2		16,5
Q04760-2	Isoform 2 of Lactoylglutathione lyase	17,75		2		19,0
I3L4X3	NF-kappa-B inhibitor beta	17,52			3	25,2
B7ZB63	ADP-ribosylation factor 3	17,36	2	2	1	16,1
B4DTG6	Protein LSM14 homolog A	17,30	1	4	2	46,4
B7Z972	Protein-L-isoaspartate O-methyltransferase	17,19		2		20,7
F8WDD7	Actin-related protein 2/3 complex subunit 4	16,81	2	1		13,0
P60842	Eukaryotic initiation factor 4A-I	16,75	4	2	2	46,1
Q5QNY5	Peroxisomal biogenesis factor 19	16,60	1		2	26,1
P51148	Ras-related protein Rab-5C	16,20		3	1	23,5
H7C488	Leucine zipper transcription factor-like protein 1	16,17	1	1	1	26,9
Q99832-3	Isoform 3 of T-complex protein 1 subunit eta	15,63	3	3	5	54,8
F5H7C6	COP9 signalosome complex subunit 7a	15,63		1	1	21,3
E9PM17	Serine/threonine-protein kinase PAK 1	15,60		2	3	50,8
D6RE83	Ubiquitin carboxyl-terminal hydrolase isozyme L1	15,46		5		23,2
P62266	40S ribosomal protein S23	15,38	3	3	1	15,8
I3L2E3	Leucine carboxyl methyltransferase 1	15,05		2		10,6
E9PH29	Thioredoxin-dependent peroxide reductase	14,71	1	2		25,8
H0YMH7	CTD small phosphatase-like protein 2	14,55		2		18,3

Acesso	Descrição	ΣCoverage	Amostra 1	Amostra 2	Amostra 3	MW [kDa]
P17987	T-complex protein 1 subunit alpha	13,49		4	1	60,3
E9PM04	DCN1-like protein 5	13,16		2		17,8
Q96F86	Enhancer of mRNA-decapping protein 3	12,99	3	2		56,0
P46777	60S ribosomal protein L5	12,12	2		1	34,3
B4E3P0	ATP-citrate synthase	11,93	5		4	91,0
Q12962	Transcription initiation factor TFIID subunit 10	11,47	2	1	1	21,7
H0YCP8	Poly(U)-binding-splicing factor PUF60	11,36		1	1	28,1
P62820-2	Isoform 2 of Ras-related protein Rab-1A	11,35		1	1	15,3
Q9NYB0	Telomeric repeat-binding factor 2-interacting protein 1	11,28	1		1	44,2
Q9H444	Charged multivesicular body protein 4b	10,71		1	1	24,9
O43242	26S proteasome non-ATPase regulatory subunit 3	10,67	2	2	3	60,9
Q9Y6I3-3	Isoform 3 of Epsin-1	10,55	2	1		57,5
Q93062-4	Isoform D of RNA-binding protein with multiple splicing	10,49	1	1	1	15,8
O75446	Histone deacetylase complex subunit SAP30	10,45	2	1	1	23,3
Q96G25	Mediator of RNA polymerase II transcription subunit 8	10,45			2	29,1
P41250	Glycine--tRNA ligase	10,42	3	1	2	83,1
Q9Y371	Endophilin-B1	10,41	2	1		40,8
Q96IZ0	PRKC apoptosis WT1 regulator protein	10,29	1		2	36,5
P17028	Zinc finger protein 24	10,05		1	2	42,1
Q68CR9	Aspartate--tRNA ligase	9,48	1		1	45,7
B4DME9	Kinesin light chain 4	9,41		2	1	59,6
B4DNE4	Septin-7	9,16	1	3		44,6
I3L1P5	Hepatocyte growth factor-regulated tyrosine kinase substrate	9,13		2	1	30,0
Q12846	Syntaxin-4	9,09	1		1	34,2
O75351	Vacuolar protein sorting-associated protein 4B	9,01	1	2	3	49,3
Q9Y265	RuvB-like 1	8,99	3		1	50,2

Acesso	Descrição	ΣCoverage	Amostra 1	Amostra 2	Amostra 3	MW [kDa]
Q9HBM1	Kinetochore protein Spc25	8,93			2	26,1
Q8WWZ3-2	Isoform B of Ectodysplasin-A receptor-associated adapter protein	8,78			2	23,7
O43776	Asparagine--tRNA ligase	8,76	1	3	1	62,9
O14773-2	Isoform 2 of Tripeptidyl-peptidase 1	8,75		2		34,4
O60784-3	Isoform 3 of Target of Myb protein 1	8,72		1	1	48,6
P29084	Transcription initiation factor IIE subunit beta	8,59		2		33,0
O15446	DNA-directed RNA polymerase I subunit RPA34	8,24	2		2	55,0
Q8TBZ6	tRNA methyltransferase 10 homolog A	7,96	1	1		39,7
D6RHW1	Transcription initiation factor TFIID subunit 9	7,84	1	1	1	11,6
B7Z848	DNA-binding protein RFX5	7,81	1		2	60,5
Q96BD8	Spindle and kinetochore-associated protein 1	7,45	1	1	1	29,5
O00232-2	Isoform 2 of 26S proteasome non-ATPase regulatory subunit 12	7,34	2			50,5
P49643	DNA primase large subunit	7,27	1		2	58,8
P26196	Probable ATP-dependent RNA helicase DDX6	7,25	3	2	2	54,4
Q8WXA9-2	Isoform 2 of Splicing regulatory glutamine/lysine-rich protein 1	7,21		1	2	71,6
P62851	40S ribosomal protein S25	7,20	1		1	13,7
P38919	Eukaryotic initiation factor 4A-III	7,06	2	2	1	46,8
H0YA83	Beta-hexosaminidase subunit beta	7,06		1	1	19,5
F5GY39	WASH complex subunit FAM21B	7,06			2	55,4
Q9BT09	Protein canopy homolog 3	6,83	1		1	30,7
Q9UBB5	Methyl-CpG-binding domain protein 2	6,81	1	1	1	43,2
Q9NQA3	WAS protein family homolog 6	6,71	1		1	48,0
B7Z7N2	Conserved oligomeric Golgi complex subunit 2	6,56		2		48,1
P53367-2	Isoform A of Arfaptin-1	6,45	1	1		38,6
F5H897	Heat shock protein 75 kDa	6,45	3	4	2	74,2
Q99460-2	Isoform 2 of 26S proteasome non-ATPase regulatory subunit 1	6,29	1	2	1	102,2

Acesso	Descrição	ΣCoverage	Amostra 1	Amostra 2	Amostra 3	MW [kDa]
Q9UGY1	Nucleolar protein 12	6,10		2		24,6
B4DP52	HCG2005638, isoform CRA_c	6,00	1	2	1	40,6
Q96CT7	Coiled-coil domain-containing protein 124	5,83	1	1		25,8
Q53GG5-2	Isoform 2 of PDZ and LIM domain protein 3	5,70		1	1	34,3
A6NGX6	DNA-directed RNA polymerase III subunit RPC7-like	5,64	1	1		22,6
P46783	40S ribosomal protein S10	5,45		1	1	18,9
Q9NR30-2	Isoform 2 of Nucleolar RNA helicase 2	5,17	1	2	1	79,6
Q15398-3	Isoform 3 of Disks large-associated protein 5	5,11	2		1	94,1
Q9BTT6	Leucine-rich repeat-containing protein 1	4,96		2	1	59,2
Q9H0G5	Nuclear speckle splicing regulatory protein 1	4,66	1	3	1	66,4
H0YJX6	Protein arginine N-methyltransferase 5	4,66		2		32,3
C9JW01	Pumilio homolog 2	4,44		1	1	32,0
Q7Z7F0-2	Isoform 2 of UPF0469 protein KIAA0907	4,43	1		1	59,1
O60841	Eukaryotic translation initiation factor 5B	4,34		4	1	138,7
Q96CN9	GRIP and coiled-coil domain-containing protein 1	4,00		1	1	87,8
O75976	Carboxypeptidase D	3,62			2	152,8
O43143	Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	3,52	2		1	90,9
Q9Y5B9	FACT complex subunit SPT16	3,44	1	1	1	119,8
Q15029-2	Isoform 2 of 116 kDa U5 small nuclear ribonucleoprotein component	3,31	1	1		105,3
Q13823	Nucleolar GTP-binding protein 2	3,15	1	2		83,6
O95677-3	Isoform 3 of Eyes absent homolog 4	3,10	1		1	48,7
F5H0N7	CAP-Gly domain-containing linker protein 1	3,06			2	116,2
Q9BQ52-4	Isoform 4 of Zinc phosphodiesterase ELAC protein 2	2,80	1	1		87,5
H3BRV0	Eukaryotic translation initiation factor 3 subunit C	2,66		2	1	104,0
P49756	RNA-binding protein 25	2,61		1	1	100,1
P56192	Methionine--tRNA ligase	2,56	1		1	101,1

Acesso	Descrição	ΣCoverage	Amostra 1	Amostra 2	Amostra 3	MW [kDa]
P29144	Tripeptidyl-peptidase 2	2,16		2	1	138,3
Q08211	ATP-dependent RNA helicase A	2,05	1		1	140,9
B0V043	Valine--tRNA ligase	1,82		1	1	140,4
E9PFK5	Nucleolar protein 14	1,78	1	1	1	88,8
P49327	Fatty acid synthase	1,59	1		2	273,3
H3BQQ2	Zinc finger protein 598	1,41	2		1	93,2
Q96RT1-7	Isoform 7 of Protein LAP2	0,92	1		1	146,0

Os experimentos de imunoprecipitação permitiram a identificação de 225 proteínas totais capazes de imunoprecipitar com hNek5. Destas, 92 estavam presentes nas frações mitocondriais e 133 nas frações citoplasmáticas. Para identificação destas proteínas foi utilizado como critério de seleção para eliminação de falsos positivos filtros bastante rígidos e por isso proteínas identificadas no duplo híbrido não foram confirmadas por espectrometria de massas, o que não invalida nenhuma das duas abordagens, apenas agrega resultados.

Algumas das proteínas identificadas já foram caracterizadas como proteínas capazes de interagir com outros membros da família das Neks. Como por exemplo, a proteína Fez-1 que interage com Nek1 (Lanza et al., 2010). Apesar de não ser classificada como uma proteína mitocondrial, Fez1 está envolvida no processo de transporte de mitocôndrias. A proteína FEZ1 foi caracterizada inicialmente como um ortólogo da proteína UNC76 de *C. elegans*, responsável pelo desenvolvimento e fasciculação neuronal nesse verme (Bloom & Horvitz, 1997). Fez1 está envolvida em processos de desenvolvimento neuronal (Miyoshi et al., 2003; Honda et al., 2004), polarização celular (Ikuta et al., 2007), mecanismos de transporte associado à kinesinas (Bloom & Horvitz 1997; Gindhart et al., 2003) e transporte de vesículas e mitocôndrias (Suzuki et al., 2005; Fujita et al., 2007). FEZ1 é capaz de interagir com mais de 51 proteínas diferentes, e participa em muitos processos celulares. Sua capacidade de interagir com outras proteínas é influenciada pela fosforilação da sua região C-terminal por diferentes isoformas de PKC. FEZ1 interage e colocaliza com NEK1 em uma região candidata ao centrossomo (Lanza et al., 2010). Essas interações são dependentes da região *coiled-coil* presente na parte C-terminal de FEZ1, e ocorrem em regiões *coiled-coil* de NEK1. Baseado nestes dados Nek5 apresenta regiões *coiled coil* que podem ser investigadas como potenciais sítios de interação com Fez1.

Outras proteínas identificadas como potenciais interatores de hNek5 são membros de famílias que interagem com outras Neks como é o caso de PPP1R7 (Protein phosphatase 1 regulatory subunit 7), PRDX6 (Peroxiredoxin-6) e CAP1 (Adenylyl cyclase-associated protein 1) que foram identificadas na imunoprecipitação. Nek1 também interage com PPP2R5A/D (protein phosphatase 2A) através de regiões de *coiled coil* (Surpili et al., 2003). A PPP2R5A é uma Ser/Thr fosfatase com elevada expressão

em cérebro e está relacionada com o controle negativo de células em crescimento e divisão (Gentry & Hallberg, 2002). Já a PPP1R7 é uma proteína de 360 aa fortemente correlacionada com a vulnerabilidade regional associada a doença de Huntington (Lewandowski et al., 2003). Ela também está relacionada a regulação da polaridade e forma em células epiteliais (Grusche et al., 2009), além de regular a atividade da aurora B e interações microtúbulos-cinetocoro (Posch et al., 2010). Funções relacionadas a microtúbulos são fortemente relacionadas as Neks. Identificada na fração mitocondrial da imunoprecipitação a peroxiredoxina 6 está envolvida na regulação redox celular e pode ter um importante papel na regulação do *turnover* de fosfolipídeos, assim como na proteção contra a injúria oxidativa (Manevich *et al.*, 2005). Meirelles e colaboradores demonstraram em 2010 a interação entre hNek6 e PRDX3.

A ontologia das proteínas identificadas foi realizada pelo software *Integrated Interactome System* (desenvolvida no LNBio). Esta plataforma é baseada na anotação, análise e visualização do perfil de interação de proteínas/genes de interesse. Este programa realiza uma busca em diferentes bancos de dados para redes de interações como GPGDIN, Intact, DIP, String, MINT e HPRD criando uma rede de interações entre as proteínas (Carazzolle et al., 2014). As figuras 10 e 11 representam as proteínas identificadas e classificadas de acordo com processos biológicos envolvidos e localização celular.

A estratégia de fracionamento foi eficiente para o enriquecimento em proteínas mitocondriais. Porém o fracionamento não foi 100% puro, pois, imagens de *western blot* apresentadas no artigo II permitiram identificar pequenas quantidades de marcadores citoplasmáticos nas frações mitocondriais. Como o objetivo deste trabalho era o enriquecimento em informação sobre proteínas capazes de interagir com hNek5 a pureza de amostras não foi fundamental para atingir o objetivo proposto. Grande parte das proteínas identificadas são proteínas citoplasmáticas, mas, é possível observar uma significativa porção de proteínas mitocondriais (Fig11).

Muito ainda precisa ser feito para a elucidação das funções de hNek5. Até o presente momento, apenas um artigo científico sugere o papel desta proteína no processo de diferenciação como substrato para caspase 3 (Shimizu et al., 2013). A ampliação do conhecimento de parceiros de interação da hNek5 poderá desvendar as vias de

sinalização e os mecanismos funcionais aos quais a Nek5 pode estar envolvida. A figura 10 representa as proteínas identificadas por espectrometria de massas e classificadas de acordo com seu papel biológico. Uma proteína pode estar associada a mais de uma função por isso utilizamos a plataforma *Integrated Interactome System* para o enriquecimento das proteínas em processos biológicos comuns à várias destas. É possível observar que muitas das proteínas identificadas estão envolvidas em processos tais como: expressão gênica, transcrição, tradução, apoptose e morte celular. O envolvimento de hNek5 no processo de morte celular/ apoptose foi investigado neste trabalho e os resultados obtidos pode ser visualizados no artigo II deste trabalho. Os demais processos enriquecidos podem sugerir um papel da hNek5 na transcrição / tradução e expressão gênica abrindo perspectivas para novos estudos.

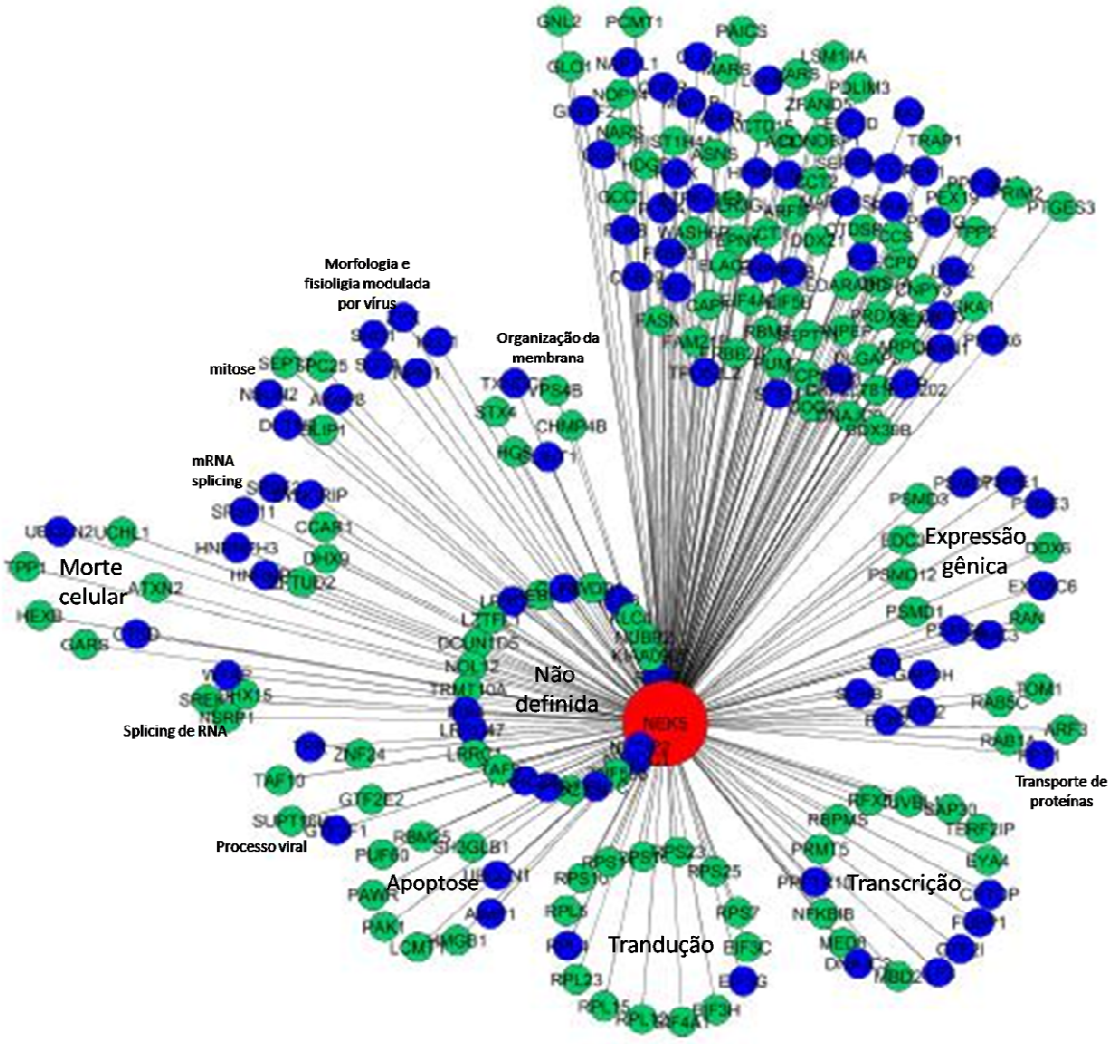


Figura 10: Rede de interações da Nek5 humana. Células FLPIn que expressam estavelmente a hNek5-FLAG foram fracionadas em citoplasma e mitocôndria.As proteínas foram submetidas a imunoprecipitação em coluna contendo Anti-FLAG imobilizado. As proteínas imobilizadas foram separadas por SDS-PAGE e identificada por espectrometria de massas. A rede possui 133 proteínas identificadas em frações de citoplasma (nodos verdes) e 92 proteínas identificadas nas frações de mitocôndrias (nodos azuis). A rede foi organizada de acordo com os processos biológicos (GeneOntology) em que as proteínas identificadas estão envolvidas. A rede foi gerada utilizando o programa *Cytoscape 2.7*.

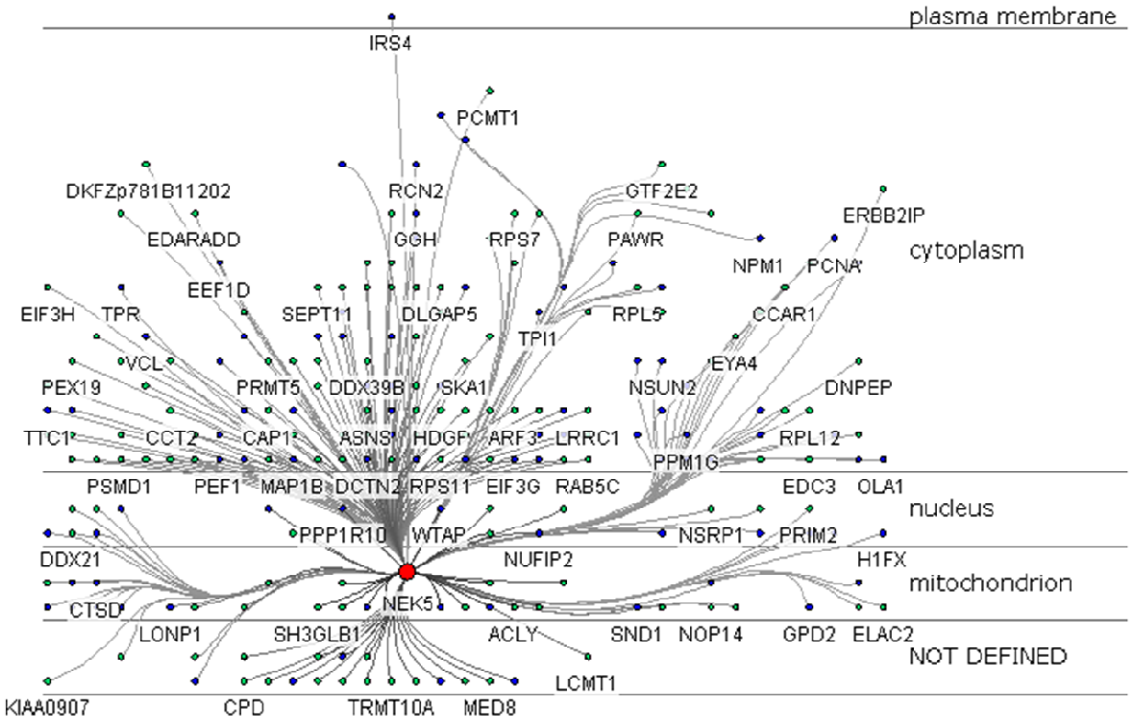


Figura 11: Localização das proteínas da rede de interações da Nek5 humana. As proteínas identificada por espectrometria de massas foram organizadas quanto a sua localização celular, de acordo com o GeneOntology. Proteínas de organelas foram organizadas na fração citoplasmática e apenas a mitocôndria foi utilizada nesta abordagem. A rede possui 133 proteínas identificadas em frações de citoplasma (nodos verdes) e 92 proteínas identificadas nas frações de mitocôndrias (nodos azuis). A rede foi gerada no programa *Cytoscape 2.7* utilizando o módulo de extensão *Cerebral v1.2*.

Como apresentado no artigo III deste trabalho a Nek5 interage e fosforila TTLL4 inativando-a e consequentemente diminuindo a poliglutamilação de proteínas. van Dijk e colaboradores (2008) realizaram imunoprecipitação de proteínas poliglutamiladas utilizando anticorpo anti-GT335 (reconhecem cadeias grandes de glutamato) e identificaram as proteínas por espectrometria de massas. Além de tubulinas outras proteínas foram identificadas como imunoprecipitando com GT335, sugerindo que sejam poliglutamiladas. A IP é capaz de detectar além de interações diretas também interações indiretas, uma vez que complexos proteicos podem ser co-imunoprecipitados. Dessa forma, analisamos as proteínas obtidas em nosso experimentos de imunoprecipitação de Nek5 com as proteínas obtidas por van Dijk e colaboradores (2008). Na tabela 6 pode-se observar que diversas proteínas foram observadas em ambos os experimentos, refuntando os dados de espectrometria, bem como a interação entre Nek5 e TTLL4. A proteína TTLL4 é a principal enzima responsável pela poliglutamilação de substratos não tubulínicos. Dentre as proteínas

identificadas destaca-se a proteína NAP1L1 (Nucleosome assembly protein 1-like 1) que é um conhecido substrato de TTLL4.

Tabela 6: Proteínas interatoras de Nek5 e potenciais substratos para poliglutamilação. Proteínas identificadas por espectrometria de massas após imunoprecipitacao de Nek5-FLAG e que foram identificadas como pontenciais alvos de poliglutamilação por van Dijk et al (2007).

Accession		Description	MW [kDa]	calc. pI
C9JH18	nucleoporin	PERQ amino acid-rich with GYF domain-containing protein 2	16,8	9,79
E5RHC7	EIF3H	Eukaryotic translation initiation factor 3 subunit H	1	4,3
Q12906-5	ILF3	Isoform 5 of Interleukin enhancer-binding factor 3	74,6	8,24
Q08211	DHX9	ATP-dependent RNA helicase A	1	140,9
P17987	TCP1	T-complex protein 1 subunit alpha	1	60,3
Q9Y265	RUVBL1	RuvB-like 1	1	50,2
F8W020	NAP1L1	Nucleosome assembly protein 1-like 1	24,4	4,51
H3BU31	RPL4	60S ribosomal protein L4	19,0	11,72
P52597	HNRNPF	Heterogeneous nuclear ribonucleoprotein F	45,6	5,58
Q8WXX5	DNAJC9	DnaJ homolog subfamily C member 9	3	29,9
P04406	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	36,0	8,46
P31942-3	HNRNPH3	Isoform 3 of Heterogeneous nuclear ribonucleoprotein H3	31,5	7,31
P46777	RPL5	60S ribosomal protein L5	1	34,3
Q96AG4	LRRC59	Leucine-rich repeat-containing protein 59	34,9	9,57
Q5VU61	TPM3	Tropomyosin alpha-3 chain	26,3	4,84
Q96CT7	CCDC124	Coiled-coil domain-containing protein 124	25,8	4,00
P46821	MAP1B	Microtubule-associated protein 1B	270,5	4,81
E7EX53	RPL15	Ribosomal protein L15	1	15,7
P51148	RAB5C	Ras-related protein Rab-5C	1	23,5

Ensaios estruturais de membros da família das Neks

O *Structural Genomics Consortium* (SGC) é uma organização sem fins lucrativos com o objetivo de determinar a estrutura tridimensional de proteínas com relevância médica. Nos últimos seis anos o SGC foi responsável por aproximadamente 25% da produção global de estrutura de proteínas. Como uma parceria química "*open access*", empresas públicas e privadas trabalham juntas para gerar potentes e seletivos inibidores farmacológicos de proteínas humanas. Uma parceria foi realizada entre o Dr. Jörg Kobarg e o Dr. Stephan Knapp do *Structural Genomics Consortium* (SGC), Universidade de Oxford, em Oxford-UK. Durante o desenvolvimento desta tese algumas atividades foram

realizadas no laboratório do Dr. Knapp com o objetivo de aprender e aplicar a metodologia deste grande instituto para solubilizar a expressão e melhorar a purificação dos domínios cinase das proteínas da família das Neks humanas. Diversas construções foram geradas para expressão em bactérias e células de inseto, através do sistema de baculovírus, para cada uma das neks. As diferenças nas sequências são baseadas na adição de alguns aminoácidos que flanqueiam o domínio cinase. A tabela 7 apresenta o panorama global de testes de expressão e resultados obtidos pelos alunos envolvidos neste trabalho. Ao todo mais de 600 construções foram testadas em células de inseto (Sf9) e cepa bacteriana BL21-Rosetta ou Bl21(DE3)-λ-Fosfatase.

Tabela 7: Construções de neks testadas em bactéria e células de insetos no SGC, Oxford.

	Total de contruções	**Const. testadas em (bactérias)**	**Const. expressas**	**Const. testadas (SF9)**	**Const. expressas**
Nek1	33	67	26		
Nek2	14	54	11		
Nek3	89	150	3	33	8
Nek4	88	91	1	39	19
Nek5	38	26	3	14	7
Nek6	26	47	12		
Nek7	24	34	10		
Nek8	54	44	5	32	11
Nek9	84	130	0	17	5
Nek10	59	34	1		
Nek11	102	101	1	43	33

Realizei no período em que estive no SGC ensaios de expressão e purificação das Neks 1, 5, 6 e 7. Experimentos de *thermal shift* foram realizados com as proteínas nek 6 e 7 e ensaios de cristalizaçãoo com a nek1. Os testes com construções da Nek5 transformadas em BL21(DE3)R3-lambda-ppase não permitiram a purificação de quantidades suficientes de Nek5. Entretanto, Nek1, 6 e 7 apresentavam níveis mais elevedos de expressão e por isso foram analisados mais extensivamente. Um dos clones de hNek6 foi testado em BL21(DE3)R3-lambda-ppase e BL21(DE3)R3-pRARE3-Rosetta e um clone de hNek7 foi testado em BL21(DE3)R3-lambda-ppase. Após a

cromatografia de afinidade é possível observar que todas as construções apresentaram uma expressão elevada das proteínas de interesse (Fig. 12). Foram realizadas tentativas de concentração das proteínas eluídas para a aplicação em cromatografia de gel filtração, porém ocorreu precipitação da mesma. Devido à este fato as amostras diluídas foram injetadas na coluna S200 16/60 GF para remoção de impurezas. Os picos de coleta das amostras do cromatograma estão identificados na figura 12 b,c e d.

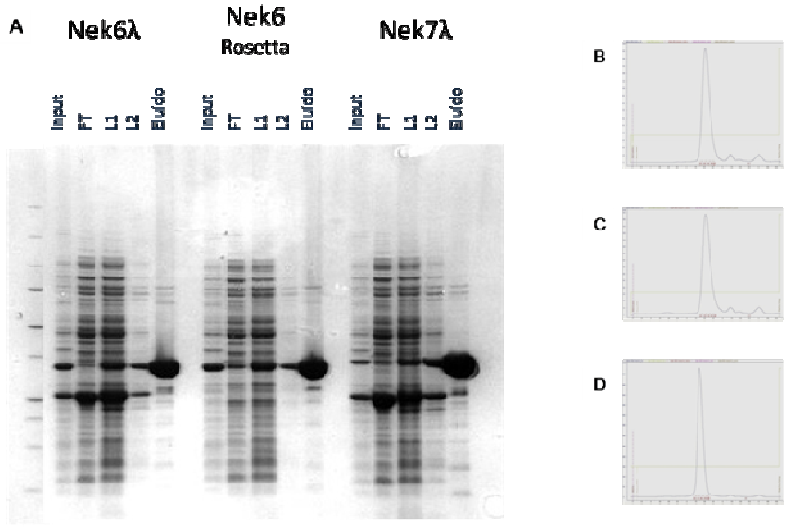


Figura 12: Teste de expressão e purificação do domínio cinase da hNek6 e hNek7. A) Gel SDS-PAGE 4-12% das frações da purificação por coluna de afinidade em coluna Ni-Sepharose. B, C e D) Picos coletados na coluna S200 16/60 de gel filtração das proteínas hNek6 em λ fosfatase, em Rosetta em hNek7 em λ fosfatase respectivamente. M= marcador de peso molecular *Precision Plus-Dual Ladder* (Bio-Rad); Input= fração solúvel da indução de expressão; FT= Fração de proteínas que não se ligaram a colun; L1= fração de proteínas liberadas após lavagem com tampão de lavagem 1; L2= fração de proteínas liberadas após lavagem com tampão de lavagem 2; Eluição= fração de proteínas liberadas após lavagem com tampão de eluição. A caixa vermelha da imagem de *Western blot* (a direita) indica a condição apresentada no relatório anterior que foi utilizada para os testes de purificação.

As proteínas hNek6 e hNek7 puras foram submetidas a técnica de "*thermal shift*" para a identificação de compostos capazes de inibir a atividade desta proteínas e que favoreçam a estabilização da proteína para ensaios cristalográficos. Apesas da estrutura de hNek7 já estar disponível o objetivo era identificar compostos que se ligassem a regiões distintas da molécula conhecida, promovendo uma conformação diferente. Para

isso, as amostras foram diluídas à uma concentração de 0,07mg/mL, misturadas com o tampão, SYPRO Orange e os compostos inibitórios na concentração de 10µM. Cerca de 300 compostos conhecidamente inibidores de cinases foram testados. Para a análise dos dados gerados, a curva de desnaturação da proteína em presença de cada composto foi comparada com o controle (proteína sem ligante = apo). Foram considerados compostos viáveis aqueles que deslocaram a Tm (Temperatura de *Melting*) da proteína para um valor entre 4°C a 10°C. Como pode ser observado nas figuras 13 e 14, nenhum composto apresentou variação próxima ou superior a 4°C e por isso estas construções não foram utilizadas em ensaios posteriores de cristalização.

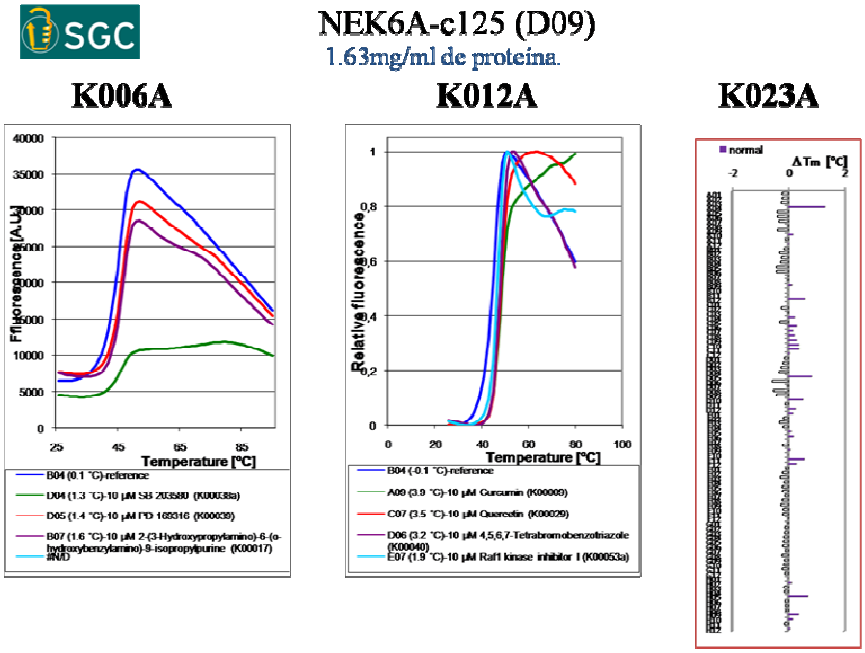


Figura 13: Thermal Shift de hNek6. O gráfico mostra o deslocamento térmico da reação após adição de alguns inibidores de cinases em relação ao composto referência (azul). A proteína foi utilizada na concentração de 1,63mg/mL em tampão 10 mM HEPES 10mM, pH 7.5 e NaCl 500 mM e diluídas para a concentração final de 2.0 µM. Foram adicionados 2uL de SYPRO-Orange (Molecular Probes) para cada 2 mL de solução. Foram realizados 71 ciclos com variação na temperatura de 20 a 100°C.

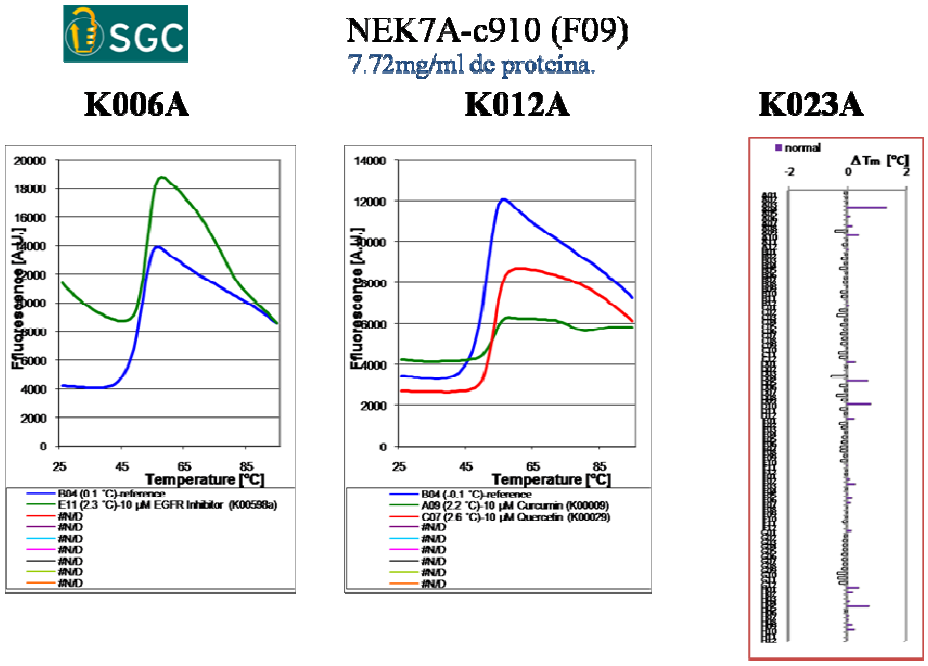


Figura 14: Thermal Shift de hNek7. O gráfico mostra o deslocamento térmico da reação após adição de alguns inibidores de cinases em relação ao composto referência (azul). A proteína foi utilizada na concentração de 7,72mg/mL em tampão 10 mM HEPES 10mM, pH 7.5 e NaCl 500 mM e diluídas para a concentração final de 2.0 µM. Foram adicionados 2uL de SYPRO-Orange (Molecular Probes) para cada 2 mL de solução. Foram realizados 71 ciclos com variação na temperatura de 20 a 100°C.

Os experimentos mais promissores ocorreram com a Nek1. Em células, a superexpressão de Nek1 inteira inibe a formação de cílios e a forma truncada, sem a região C-terminal, provoca uma perda de centrossomos (White e Quarmby, 2008. A Nek1 está envolvida na resposta precoce a danos de DNA induzidos por radiação (Polci et al., 2004) e está relacionada a doenças do Rim Policístico (Upadhya et al., 2000; Mahjoub et al., 2005). Para a compreensão da estrutura do domínio cinase da proteína humana Nek1 estudos anteriores do grupo realizaram testes de expressão e purificação de diferentes construções desta proteína. Vários clones foram capazes de expressar grandes quantidades desta proteína. Ensaios de "*Thermal Shift*" identificaram compostos capazes de inibir significativamente esta proteína, entretanto, não foram obtidos cristais capazes de refratar, ou seja, não foi possível a obtenção da estrutura desta proteína. Durante o meu período em Oxford foram testadas 4 construções diferentes de hNek1 transformadas em bactérias BL21(DE3)R3-lambda-ppase, induzidas com 0,5 mM de IPTG *overnight* a

18°C, as células foram lisadas e a fração solúvel submetida a cromatografia de afinidade por Ni-Sepharose. As amostras tiveram sua qualidade avaliada por SDS-PAGE (Fig. 15) e a fração de eluição foi concentrada para 3mLs. Para a obtenção de uma proteína com maior grau de pureza as proteínas foram injetadas na coluna S200 16/60 GF (Fig. 15B) e então novamente concentradas para aproximadamente 10mg/mL.

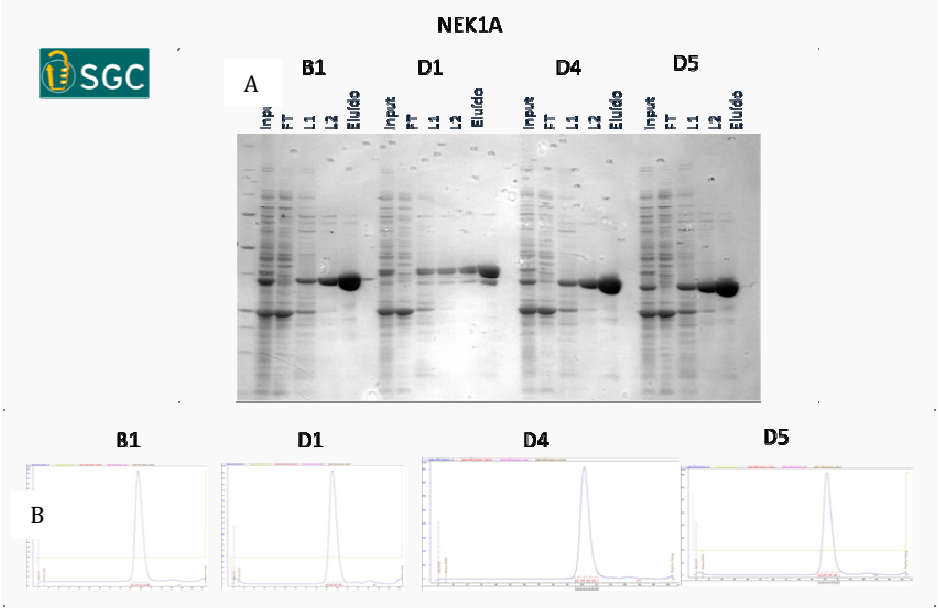


Figura 15: Teste de expressão e purificação de diferentes construções do domínio cinase da hNek1. A) Gel SDS-PAGE 4-12% das frações da purificação por coluna de afinidade em coluna Ni-Sepharose. B) Picos coletados na coluna S200 16/60 de gel filtração das diferentes construções da proteína hNek1 M= marcador de peso molecular *Precision Plus-Dual Ladder* (Bio-Rad); Input= fração solúvel da indução de expressão; FT= Fração de proteínas que não se ligaram a coluna; L1= fração de proteínas liberadas após lavagem com tampão de lavagem 1; L2= fração de proteínas liberadas após lavagem com tampão de lavagem 2; Eluição= fração de proteínas liberadas após lavagem com tampão de eluição. A caixa vermelha da imagem de *Western blot* (a direita) indica a condição apresentada no relatório anterior que foi utilizada para os testes de purificação.

As primeiras tentativas de cristalização da hNek1 foram realizadas em placas para cristal *screening* disponíveis no SGC. Estas placas apresentam diferentes concentrações de agentes tamponantes, variação de pH, presença de precipitantes como PEG, dentre

outros. O *screening* inicial apresentou alguns cristais para a construção D1, em ausência e presença de inibidores, que foram submetidas a um refinamento das condições de cristalização. A melhor condição foi 0,2M Na/KPO4, 20% PEG e 10% Etileno Glicol. Os cristais obtidos foram transportados em crioprotetores ao *Diamond Light Source* onde os dados de refração foram coletados. Os cristais da proteína hNek1 apo foram refratados com resolução de 2,2Å e de 2 Å na presença do composto k00602a. Após a volta para o Brasil houve o crescimento de um novo cristal de Nek1 em presença do composto k00546 que foi coletado e refratado eficientemente. O processamento dos dados foi realizado com o auxilio do líder de grupo, Dr. Jon Elkins utilizando os programas iMOSFLM e AIMLESS.

Na figura 16 está esquematizada a estrutura tridimensional obtida do domínio cinase da proteína hNek1. A célula unitária é formada por duas moléculas e os compostos inibitórios, k00602a e k00546, se ligam à esta proteína na região representada em branco. Baseado na estrutura da proteína hNek2 a estrutura obtida apresenta um movimento do segmento de ativação e um movimento oscilante αC. A estrutura cristalográfica da proteína hNek1 apo e na presença do composto K00602a já estão depositadas no banco de dados PDB com os códigos de identificação 4APC e 4B9D respectivamente.

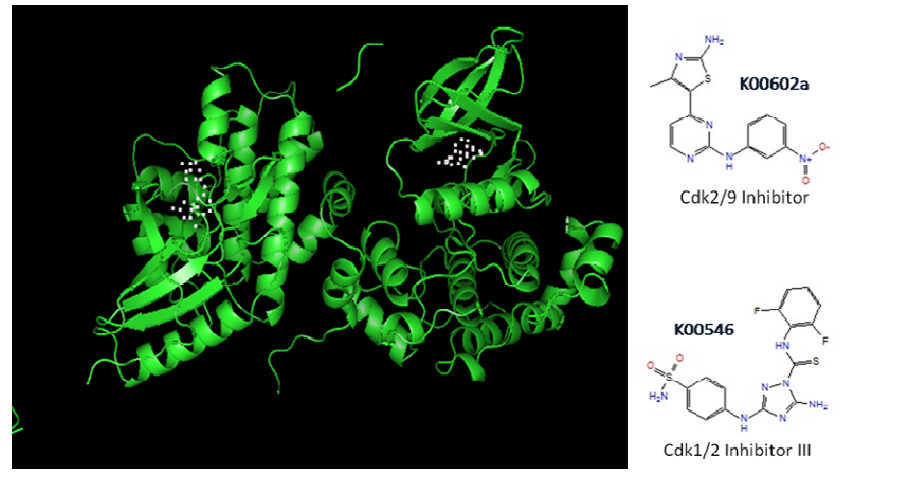


Figura 16: Estrutura cristalográfica da proteína humana Nek1 ligada ao composto K00602a. Os pontos em branco indicam o local onde os compostos indicados na figura se ligavam à estrutura tridimensional da proteína.

Declaração de autorização do Comitê de Biossegurança

Uso exclusivo da CIBio:

Número de projeto / processo: JK 6.4

Formulário de encaminhamento de projetos de pesquisa para análise pela CIBio - Comissão Interna de Biossegurança da ABTLuS – Associação Brasileira de Tecnologia de Luz Síncrotron

Título do projeto: *Expressão da proteína cinase humanas Nek5 para estudos estruturais efuncionais*
Pesquisador responsável: *Dr. Jorg Kobarg*
Experimentador: *Talita Diniz Melo*
Nível do treinamento do experimentador: [ ]-Iniciação científica, [ ]-mestrado, [X]-doutorado,
[ ]-doutorado direto, [ ]-pós-doutorado, [ ]-nível técnico, [ ]-outro,

especifique:_____ Resumo do projeto:

As proteínas quinases Nek (NIMA related kinases) constituem uma família altamente conservada de proteínas envolvidas na progressão do ciclo celular. Em humanos foram identificadas 11 Neks (1-11), sendo que para algumas a estrutura já foi determinada, bem como a relevância fisiopatológica. Várias proteínas da família das Neks são produtos de genes relativos a patologias relacionadas a defeitos na progressão do ciclo celular e no mecanismo de reparo de DNA durante a interfase, sobretudo em diferentes cânceres. A proteína Nek5 foi identificada, de acordo com sua sequência genômica em organismos como Homo sapiens, Mus musculus, Bos taurus, dentre outros. Entretanto, características inerentes a proteína permanecem pouco elucidadas, como, por exemplo, sua função, estrutura e interações. Portanto, o objetivo geral deste projeto é a clonagem, expressão e purificação de Nek5 para ensaios biofisicos e de cristalização, visando análises estruturais. Em paralelo, serão realizados: 1) estudos funcionais envolvendo silenciamento por RNA (iRNA) e superexpressão de Nek5 em células humanas; 2) identificação por espectrometria de massas de proteínas que interagem com Nek5 através de 'pull down' e ensaio de duplo híbrido; e 3) ensaios de deslocamento térmico baseado emfluorescência e outros ensaios in vitro afim de verificas alvos para inibição de atividade de Nek5.

A CIBio analisou este projeto em reunião realizada no dia: 7-·f2-....2..-

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Parecer final: [)(j-projeto aprovado, [ ]-projeto recusado, [ ]-projeto com deficiências, favor comentários abaixo:

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Presidente da CIBio - ABTLuS
Prof. Dr. Jörg Kobarg

Membro da CIBio - ABTLuS
Prof. Dr. Celso Eduardo Benedetti

Membro da CIBio
Prof. Dr. Andrea Balan